Application of Sugar Amino Acids: Flow chemistry used for α/β-chimera synthesis

Viktória Goldschmidt Gőz,[a] Kim Hoang Yen Duong,[b,c] Dániel Horváth,[b] Kristóf Ferentzi,[b,c] Viktor Farkas,[a] and **András Perczel*[a,b]**

[a] MTA-ELTE Protein Modeling Research Group, Eötvös Loránd Research Network (ELKH) ELTE Eötvös Loránd University – Pázmány P. stny. 1/A, 1117 Budapest, Hungary

[b] Laboratory of Structural Chemistry and Biology, Institute of Chemistry, ELTE Eötvös Loránd University – Pázmány P. stny. 1/A, 1117 Budapest, Hungary

[c] Hevesy György PhD School of Chemistry, Institute of Chemistry, ELTE Eötvös Loránd University – Pázmány P. stny. 1/A, 1117 Budapest, Hungary

* e-mail: perczel.andras@ttk.elte.hu

Kim Hoang Yen Duong and Viktória Goldschmidt Gőz: These authors contributed equally to this work.

ABSTRACT

Two ring size β-Sugar Amino Acids, βSAAs, Fmoc-RibAFU(ip)-OH and Fmoc-GlcAPU(Me,Bn)-OH, as Lego-elements are introduced to make α/β-chimera peptides by flow-based solid phase peptide synthesis (SPPS). Their synthesis alongside selected α-amino acids, αAA, are fine-tuned. The recently published 50% TFA cleavage protocol of tBu protected shorter (Ser, Asp) and larger aromatic (Tyr, Trp), with bulky side chain protected Arg(Pbf) and Gln(Trt) residues were probed. We found that this milder condition is sufficient to successfully remove both the 1,2-*O*-isopropylidene from RibAFU(ip) and tBu, Pbf and Trt from the other αAA residues, but to preserve the 2,3-di-*O*-benzyl protection of GlcAPU(Me,Bn). Note, that *O*-benzyl groups can be subsequently cleaved by HF or catalytic hydrogenation. Tuned protocols allow the efficient synthesis of 16-mer penetratin analogues *via* continuous flow conditions incorporating either RibAFU(ip) or GlcAPU(Me,Bn) βSAAs. Both acid concentration (50%/95%) and type (TFA/HF) allow a versatile protecting group removal and thus, to fine-tune the hydrophilicity and aromaticity of the above building blocks in chimera constructs.

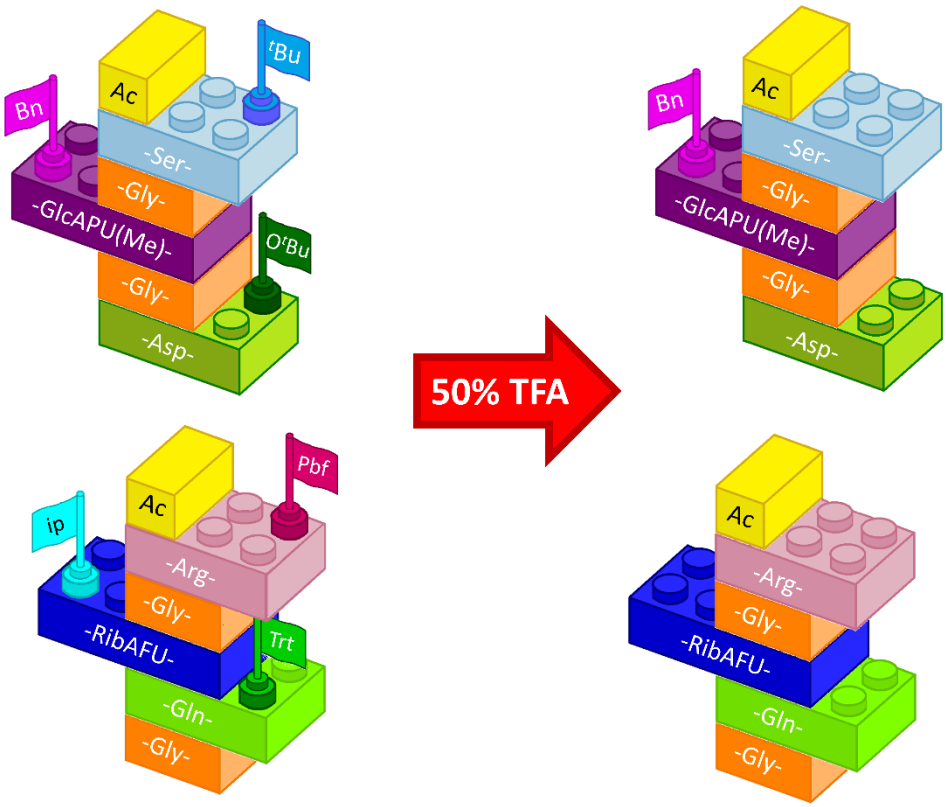
Abbreviations

αAA	α-Amino acids
Ac_2O	Acetic anhydride
ACBC	2-Aminocyclobutane-1-carboxylic acid
ACHC	2-Aminocyclohexane-1-carboxylic acid
ACPC	2-Aminocyclopentane-1-carboxylic acid
AcOH	Acetic acid
βSAA	β-Sugar Amino Acid
Boc	*tert*-Butyloxycarbonyl
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DIC	*N,N'*-Diisopropylcarbodiimide
DIEA	*N,N'*-Diisopropylethylamine
DMF	Dimethylformamide
DSS	Sodium trimethylsilylpropanesulfonate
ECD	Electronic circular dichroism spectroscopy
EDT	Ethane 1,2-dithiol
ESI	Electrospray ionization
FA	Formic acid
Fmoc-GlcAPU(Me,Bn)-OH	Methyl *N*-9-fluorenylmethoxycarbonyl-2,3-di-*O*-benzyl-4-amino-4- deoxy-α-D-glucopyranoside uronic acid
Fmoc-RibAFU(ip)-OH	1,2-*O*-Isopropylidene-*N*-(9-fluorenylmethoxycarbonyl)-3-amino-3-deoxy-α-D-ribofuranuronic acid
-GlcAPU-	4-Amino-4- deoxy-α-D-glucopyranoside uronic acid residue
-GlcAPU(Me)-	Methyl 4-amino-4-deoxy-α-D-glucopyranoside uronic acid
-GlcAPU(Me,Bn)-	Methyl 2,3-di-*O*-benzyl-4-amino-4-deoxy-α-D-glucopyranoside uronic acid
HILIC	hydrophilic interaction liquid chromatography
HPLC	High Pressure Liquid Chromatography
HF	Hydrofluoric acid
HOBt	1-Hydroxybenzotriazole
^{i}PrOH	Isopropyl alcohol
MeCN	Acetonitrile
MS	Mass Spectrometry
OtBu	*tert*-Butyl ester group
Pbf	2,2,4,6,7-pentamethyIdlhydrobenzofuran-5-sulfonyl group
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
-RibAFU(α/β)-	3-Amino-3-deoxy-α/β-D-ribofuranuronic acid residue
-RibAFU-	3-Amino-3-deoxy-α/β-D-ribofuranuronic acid residue
-RibAFU(ip)-	1,2-*O*-Isopropylidene-3-amino-3-deoxy-α-D-ribofuranuronic acid residue
RT	Room temperature
RP	Reverse phase
SPPS	Solid Phase Peptide Synthesis
tBu	*tert*-Butyl ether group
TFA	Trifluoroacetic acid
TFE	2,2,2-Trifluoroethanol
TIS	Triisopropylsilane
Trt	Trityl group
UHPLC-MS	Ultra-high-performance liquid chromatography-mass spectrometry
UV-Vis	Ultraviolet-visible spectroscopy
-XylAFU(ip)-	1,2-*O*-Isopropylidene-3-amino-3-deoxy-α-D-xylofuranuronic acid residue

Keywords: sugar amino acids, flow chemistry, foldamer, cleavage protocol, solid phase peptide synthesis, penetratin analogues

Graphical abstract

Tuned protocols including acid concentration (50%/95%) and type (TFA/HF) allow the efficient synthesis and protecting group removal from different Lego elements of β-Sugar Amino Acids, to enlarge the pool of biocompatible chimera constructs made by flow chemistry.



Introduction

Sugar amino acids, SAAs, feature the preferred properties of both amino acids and carbohydrates. The range of structural options is very large for this family due to the diversity of carbohydrates configuration and constitution. They could be grouped upon their ring size (*e.g.* furanoyl, pyranoyl), type and position of the functional groups as published in compendiums.[1,2] As SAAs are biocompatible, often biodegradable and have tunable hydrophilic/hydrophobic character, they offer a versatile applicability for modern synthetic bio- and pharmaceutical chemistry. With common proteinogenic and other natural α-amino acids, they can be suitable building blocks of chimera peptides, foldamers, glycomimetics, peptidomimetics or nucleotidomimetics.[2-12] Some SAAs are present in nature as sialic acids, neuraminic acid[13] *etc.* or bacterial cell wall components,[14,15] bacterial saccharides[16] and antibiotics.[17]

A commonly used sugar amino acid type is β-Sugar Amino Acid, βSAA, in which the carboxyl and amino function are in β-position with respect to each other.[1,4-6,18-20] Among them both five-membered ring derivatives, like D-ribo-[4] and D-xylofuranuronic acids[5,19,21] -RibAFU(ip)- and -XylAFU(ip)- and the six-membered ring derivatives, *e.g.* D-glucosamine carboxylic acid are widely used as monomeric building blocks.[3,6,18,22] These monomers can be hydrophilic analogues of the corresponding ACBC[23] (2-aminocyclobutane-1-carboxylic acid), ACPC[24] (2-aminocyclopentane-1-carboxylic acid) and ACHC[25] (2-aminocyclohexane-1-carboxylic acid) monomers, which nowadays are widely used in foldamer chemistry.[26-31]

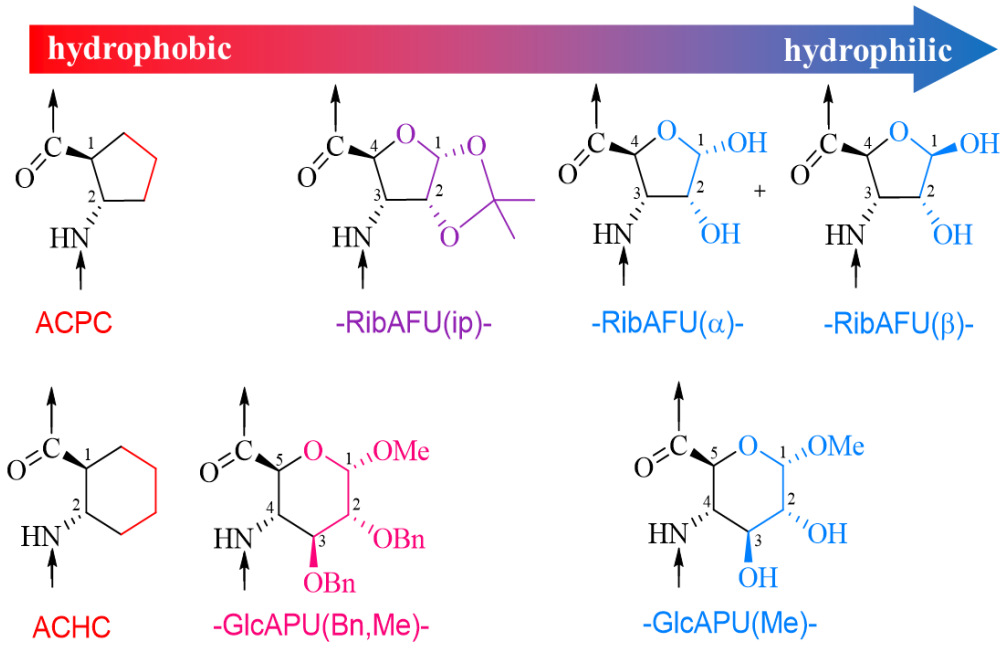


Figure 1. Furanoid and pyranoid β-sugar amino acids as hydrophilic analogues of 1,2-*trans*-ACPC and 1,2-*trans*-ACHC, the two most common hydrophobic β-amino acid residues. The RibAFU has two α- and β-anomeric form in equilibrium, -RibAFU(α)- and -RibAFU(β)-, after 1,2-*O*-isopropylidene removal. The GlcAPU

residue has temporarily 2,3-*O*-benzyl and the permanent methyl glycoside protection, the latter avoiding ring opening.

Unlike the generally used OtBu/tBu or Trt side chain protection strategy during Fmoc chemistry polypeptide synthesis, no canonized OH protection is yet accepted for βSAAs, but several alternatives coexist. The large pool comprises *O*-benzoyl,[32,33] *O*-acetyl,[18,32,34] acetonide or isopropylidene[4,5,19,21,35,36] derivatives or even free, unprotected OH could be in use[37-39] during solution phase peptide synthesis.[40] Thus, protecting, coupling and selective cleaving of chimeric oligomers remain a challenge. There are different methods to remove protecting groups before and others after the total synthesis of the oligopeptides.[38,41]

Recently, we have successfully probed two βSAAs, namely Fmoc-RibAFU(ip)-OH (**1**) and Fmoc-GlcAPU(Bn,Me)-OH (**2**) for coupling and cleaving using various coupling reagents for simple and short sequences. The ^{1}H-NMR fine-tuned and optimized synthesis of the H-Gly-**X**-**X**-Gly-OH tetrapeptide, **X** is one of the two above mentioned βSAA, was made both with preserving the side chain protecting 1,2-*O*-isopropylidene and 2,3-di-*O*-Bn groups.[42] In addition, we have recently worked out a modified protocol to cleave the 1,2-*O*-isopropylidene protecting group during the final cleavage for the model Ac-Gly-Gly-**X**-Gly-Gly-OH/NH_2 pentapeptides.[43]

Here we present the comprehensive analysis of manual and flow-based SPPS of small, but biologically relevant oligopeptides and their chimera analogues containing either the furanoid[19] (**1**) or pyranoid[20] (**2**) βSAAs. These building blocks have a tunable hydrophilicity as 1,2-*O*-isopropylidene and 2,3-di-*O*-benzyl protecting groups are now selectively removable. The recently modified 50% TFA cleavage protocol[42,43] is probed for tBu protected Ser and Asp residues, besides the larger aromatic (Tyr, Trp) and bulkier Arg(Pbf) and Gln(Trt) residues. Finally, an improved flow chemistry[44] coupling protocol is presented here to make α/β-chimera of the 16-mer penetratin, a polypeptide that overcomes the plasma membrane barrier and efficiently delivers molecular cargoes inside the cell.[45-51] These analogues and their ECD spectra show that these βSAAs are promising structure driven substitutes of hydrophilic and/or aromatic residues.

Results and discussions

Oligopeptide model selection

From a chemical point of view the applicability of our recently tuned 50% TFA cleavage protocol was probed for βSAAs (-RibAFU(ip)- and -GlcAPU(Bn,Me)-) side-by-side with tBu/OtBu or Boc protected (Ser, Asp, Tyr or Trp) and the bulkier Arg(Pbf) and Gln(Trt) αAA residues. Biologically relevant pentapeptides were selected using ProteinBlast[52] to get H-αAA1-αAA2-**βSAA**-αAA3-αAA4-OH type model sequences and thus, helping and guiding future synthetic efforts of the community making similar synthons. Standing for hydrophobic residues such as Leu and Met, the sequences of SG**L**GD, GW**L**YG and GW**M**YG were chosen. They all adopt a β-stranded secondary structure derived from integrin beta,[53] or intersectin[54] and nebulin,[55] respectively. The analogue of SG**Y**GD, part of Fab311 heavy chain was made by using the benzyl protected analogue -GlcAPU(Bn,Me)- replacing the Tyr/**Y** residue .[56] Ser/**S** residues of SG**S**GD (taken from PfS25 antibody)[57] and GW**S**YG (chain A of Dipeptidyl Peptidase IV)[58] were selected and replaced by hydrophilic βSAAs. All these selected pentapeptides show diverse secondary structural properties, as they adopt either an α-helix, a β-strand, bridges, loops and even can be part of intrinsically disordered structures (**Table 1**). More on proteins incorporating the above pentapeptides units selected and synthesized below are summarized in **STable1-3**.

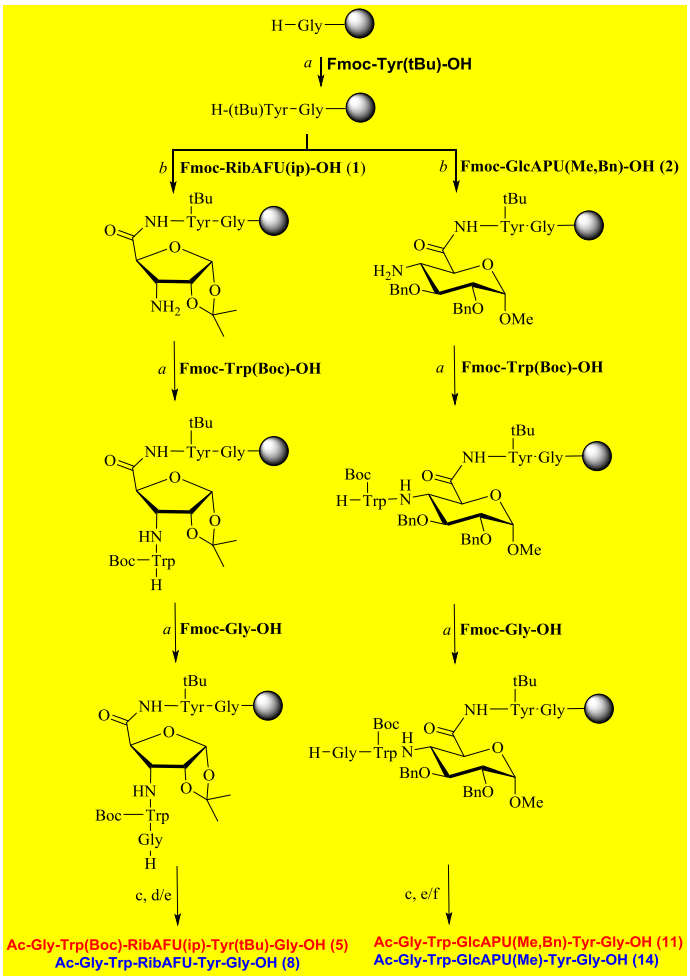
Table 1. Selected pentapeptides from proteins retrieved from ProteinBlast which adopt different secondary structural elements and incorporate αAAs with acid label side chain protecting groups as tBu, Trt and Pbf during Fmoc-SPPS.

Secondary Structure Type							
α-helical	**β-stranded**		**Bridges / loops**		**Disordered**		
RGWGQ[a]	SGLGD SGYGD SGSGD	GWLYG GWMYG	SGYGD	GWKYG GWAYG	SGLGD SGSGD SGTGD	RGVQG RGSQG RGQQG	GWGYG

a) Amino acid residues at the center (highlighted red) are replaced by βSAAs, namely either by -RibAFU(ip)- or -GlcAPU(Bn,Me)- residues.

Syntheses of model peptides

Based on the above criteria three models were constructed to test the modified coupling and cleavage protocol for βSAAs. Both Fmoc-RibAFU(ip)-OH (**1**) and Fmoc-GlcAPU(Me,Bn)-OH (**2**) as **X** residues were built into Ac-SG**X**GD-OH, Ac-RG**X**QG-OH and Ac-GW**X**YG-OH model sequences, respectively. Peptides *N*-terminus were acetylated to avoid side reactions during cleavage. For α-amino acids HOBt/DIC, for the sugar amino acids PyBOP/DIEA reagents were used for coupling, in line with our previous results.[42] Resin capacity measurements were carried out to calculate the efficacy of coupling. Various cleavage cocktails were used to get fully protected (**3-5**) -RibAFU(ip)- containing peptides, *O*-benzyl protected -GlcAPU(Me,Bn)- containing chimera (**9-11**) or fully unprotected oligomers with both βSAAs (-RibAFU-: **6-8,** -GlcAPU(Me)-: **12-14**) (**STable 4** and **5** and **Scheme 1**).



Scheme 1. Solid phase peptide synthesis of Ac-GW**X**YG-OH pentapeptides on 2-Cl-Trt-Cl resin, where **X**: -RibAFU(ip)- or -GlcAPU(Me,Bn)-. Reagents and conditions: ***a*** *i)* DIC (3 eqv)/HOBt (3 eqv), RT, 1 hour; *ii)* Piperidine (2%), DBU (2%), DMF, (3+17) min. ***b*** *i)* PyBOP (3 eqv)/DIEA (6 eqv), RT, 3 hours; *ii)* Piperidine (2%), DBU (2%), DMF, (3+17 or 10+40) min. ***c*** Ac_2O:DIEA:DMF (*v/v/v*, 1:1.2:3), 45 min. ***d*** AcOH:MeOH:DCM (*v/v/v*, 1:1:8), RT, 3 hours. ***e*** TFA (50%)/DCM (45%)/TIS (2.5%)/H_2O (2.5%), *v/v/*, RT, 3 hours. ***f*** HF

The two βSAAs have very different side chain protecting groups, likely to influence their coupling efficacy. As expected, due to the rigidity of the 5-membered fused-rings structure of Fmoc-RibAFU(ip)-OH, it has a lower coupling efficacy, varying between 55 and 83% (**Table 2**). Furthermore, despite of the two spacious *O*-benzyl protecting groups of the Fmoc-GlcAPU(Me,Bn)-OH, this βSAA has an elevated internal flexibility with respect to the pyranoid ring and thus, this sugar moiety shows a better coupling efficacy: 66-93%. Interestingly, coupling of the forthcoming residue to the liberated NH_2-βSAA is unaffected by the conformational and structural properties of βSAAs as all coupling efficacies were >94%, some are even close to 100%.

Table 2. Coupling efficacy of Fmoc-RibAFU(ip)-OH (**βSAA1**) and Fmoc-GlcAPU(Me,Bn)-OH (**βSAA2**) during solid phase peptide synthesis of the 3 models[a] using 2-Cl-Trt-Cl resin. (For coupling order see **Scheme 1**)

SPPS \ Model pentapeptide		SGXGD		RGXQG		GWXYG	
		βSAA1	**βSAA2**	**βSAA1**	**βSAA2**	**βSAA1**	**βSAA2**
2-Cl-Trt-Cl resin	Coupling with	Fmoc-Asp(OtBu)-OH		Fmoc-Gly-OH		Fmoc-Gly-OH	
	Resin capacity (mmol/g)	0.28	0.23	0.38	0.56	0.56	0.56
H-αAA$_1$-resin	Coupling with	Fmoc-Gly-OH		Fmoc-Gln(Trt)-OH		Fmoc-Tyr(tBu)-OH	
	Residual capacity (mmol/g)	0.27	0.23	0.33	0.51	0.41	0.40
	Efficacy of coupling (%)	96	<99	87	91	73	71
H-αAA$_2$-αAA$_1$-resin	Coupling with	**βSAA1**	**βSAA2**	**βSAA1**	**βSAA2**	**βSAA1**	**βSAA2**
	Residual capacity (mmol/g)	0.17	0.19	0.18	0.34	0.34	0.37
	Efficacy of coupling (%)	63	83	55	66	83	93
H-βSAA-αAA$_2$-αAA$_1$-resin	Coupling with	Fmoc-Gly-OH		Fmoc-Gly-OH		Fmoc-Trp(Boc)-OH	
	Residual capacity (mmol/g)	0.16	0.18	0.17	0.34	0.33	0.36
	Efficacy of coupling (%)	94	94	94	<99	97	98
H-AAA$_3$-βSAA-αAA$_2$-αAA$_1$-resin	Coupling with	Fmoc-Ser(tBu)-OH		Fmoc-Arg(Pbf)-OH		Fmoc-Gly-OH	
	Residual capacity (mmol/g)	0.15	0.18	0.17	0.34	0.27	0.36
	Efficacy of coupling (%)	94	<99	<99	<99	82	<99
Fmoc-αAA$_4$-αAA$_3$-βSAA-αAA$_2$-αAA$_1$-resin	**Overall efficacy (%)**	**54**	**78**	**45**	**61**	**48**	**64**

a) The *N*-terminus of the model pentapeptides were acetylated after removing the "last" Fmoc-group

During manual synthesis 2-Cl-Trt-Cl resin was selected, because of its versatile nature with respect to the cleaving acid type and concentration. Thus, cleaving with 50% TFA results in the fully unprotected, -RibAFU- containing sequences, such as the Ac-Gly-Trp-RibAFU-Tyr-Gly-OH (**8**) (**Scheme 1**). However, when using milder conditions, AcOH:MeOH:DCM 1:1:8 (*v/v/v*) instead of the diluted TFA, the fully protected chimera oligopeptides (**3-5**) are obtained. For example, Ac-Gly-Trp(Boc)-RibAFU(ip)-Tyr(tBu)-Gly-OH (**5**) was used latter on as a reference compound (**Scheme 1**) with respect to the fully deprotected oligopeptides (**6-8**).

To preserve the *O*-benzyl protecting groups of -GlcAPU(Me,Bn)- residues, the use of lower TFA concentration was necessary with various scavengers. Due to the presence of Arg and Trp, both sensitive residues to acid, the commonly used protocol[59] was applied with TFA concentration reduced to 50%. We found that for the -RibAFU- containing oligopeptides (**6**-**8**) this reduced (50%) TFA content is not effective enough, neither in the DCM (35.3%)/H_2O (4.2%)/phenol crystal (4.2%)/thioanisol (4.2%)/EDT (2.1%), nor in the DCM (45%), EDT (2.5%) and H_2O (2.5%) mixtures. The expected products were not obtained. Fortunately, the modified mixture comprising 50% TFA in DCM (45%), H_2O (2.5%) and TIS (2.5%) was found suitable to remove the 1,2-*O*-isopropylidene protection from -RibAFU(ip)- residue. Moreover, this mixture is effective enough to remove all the other side chains protecting groups as well, namely Pbf, Trt and tBu from the αAAs, but avoids the *O*-benzyl deprotection of GlcAPU(Bn,Me). Furthermore, for this pyranoid βSAA the scavengers like EDT, phenol, thioanisol or TIS using for the various cleavage cocktails, had no effect on the purity of the crude peptides (**9**-**11**). The benzyl protection of -GlcAPU(Me,Bn)- moiety was removed after the final cleavage with HF, or from Ac-SG**X**GD-OH (**9**) peptide with catalytic hydrogenation in H-Cube® reactor, using the optimized 80°C, 50 bar and 0.5 ml/min flow rate with recirculation or in an autoclave with 10 bar H_2 pressure at room temperature. In this way, the hydrophilic or fully unprotected chimera peptides (**12**-**14**) were successfully obtained (see **STable 5**).

Characterization of the model peptides

Both crude and purified oligopeptides were characterized with HPLC, MS or UHPLC-MS. Fully protected (**3**-**5**) –RibAFU(ip)- containing, *O*-benzyl protected (**9**-**11, 13**-**14**) -GlcAPU(Me,Bn)- containing and one of the unprotected -RibAFU- containing (**8**) pentapeptides were analyzed by RP-HPLC using a C18 column. As the fully unprotected chimera, both Ac-SG**X**GD-OH (**6**, **12**) and the -RibAFU- containing Ac-RG**X**QG-OH are very hydrophilic (**7**) HILIC column was used for HPLC. All analytical data are summarized in **STable 4** for -RibAFU- and **STable 5** for -GlcAPU(Me)- containing chimera peptides.

The removal of the 1,2-*O*-isopropylidene protecting group from **-RibAFU(ip)-** within the oligopeptide resulted in the α/β-anomeric mixture of the chimera. The Ac-GW**X**YG-OH was selected to study such an anomeric mixture, as in water the α/β-anomers are always in equilibrium *via* their open-form. Therefore, they are inseparable from each other and thus, both furanoyl anomers, unlike their open-forms are to be observed. Even though, during HPLC chromatography the anomeric mixture gives a single peak (**Figure 2a**) running ^{1}H-NMR on their anomeric mixture makes possible to identify the appropriate resonance frequencies belonging either to the α- or β-anomers. Resonances assigned by 2D-NMR for chimera Ac-Gly-Trp-**RibAFU(α/β)**-Tyr-Gly-OH (**8**) show a ratio of α:β/7:3 by comparing the

^{1}H signal's integrals of the ring H1 protons, assigned as 5.34 and 5.29 ppm, respectively (**Figure 2b, STable 6**).

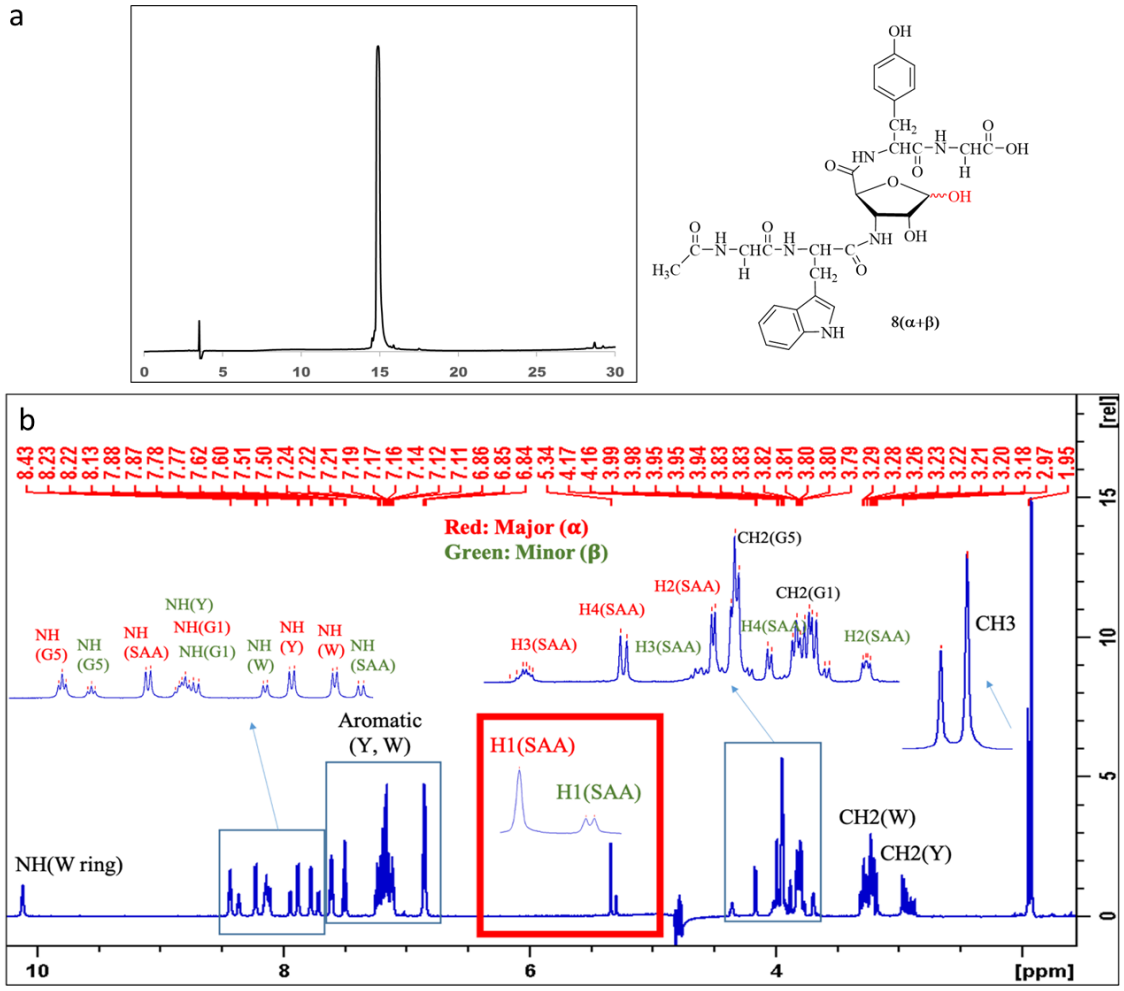


Figure 2. a) The HPLC chromatogram of the pure Ac-Gly-Trp-**RibAFU(α/β)**-Tyr-Gly-OH (**8**) chimera in a C18 column. The two α/β-anomers have the same retention time (15.0 min) and thus, the anomers are in rapid equilibrium. **b)** ^{1}H-NMR spectrum of Ac-Gly-Trp-**RibAFU(α/β)**-Tyr-Gly-OH (**8**) chimera showing selective resonances of the major (α) and minor (β) anomeric moieties. The ratio of the two anomers is 7:3/α:β/major/minor determined by using the H1 ring proton resonances, 5.34 and 5.29 ppm, respectively. ^{1}H-NMR spectrum was recorded at 700 MHz, using 0.375 mg sample dissolved in 90% H_2O (270 µl) + 10% D_2O (30 µl) + DSS/azid (3 µl) at *T*= 298 K.

The fully protected Ac-Gly-Trp(Boc)-**RibAFU(ip)**-Tyr(tBu)-Gly-OH (**5**) was made and used as the reference structure for the anomeric mixture of chimera **8**. Both HPLC and MS data revealed the fully protected oligopeptide (**5**) as a single and pure component (**SFigure 10**). 2D-NMR data revealed that the ^{1}H signal of the ring H1 resonance of **5** is at 5.92 ppm (**SFigure 1** and **STable 7**), and it is a single component only.

Flow synthesis of the model peptides

Once both coupling and cleaving conditions for flow chemistry were optimized, all six pentapeptides were resynthesized.[44] High temperature (80 °C) and high pressure (6-7 MPa), combined with our recently fine-tuned protocols allow a fast, efficient and automated total synthesis. Using a column selector valve all six chimera were set up at one go (**SFigure 25**). For synthesis 150 mg RAM-Tentagel® resin was used, resulting in *C*-amidated oligopeptides: **15-20**. The α-amino acids were coupled by using αAA protocol (12 min cycle time, 0.3 ml/min flow rate), while for βSAA a longer coupling protocol was developed. The latter one request in total a 26 min long cycle time, at a reduced flow rate of 0.05ml/min, which gives an enhanced coupling efficacy (**Figure 3**). Cleavage was achieved by using 50% TFA in DCM (45%), H_2O (2.5%) and TIS (2.5%) or for chimera Ac-GW-**GlcAPU(Me,Bn)**-YG-NH_2, TIS was changed to EDT to get a higher product purity.

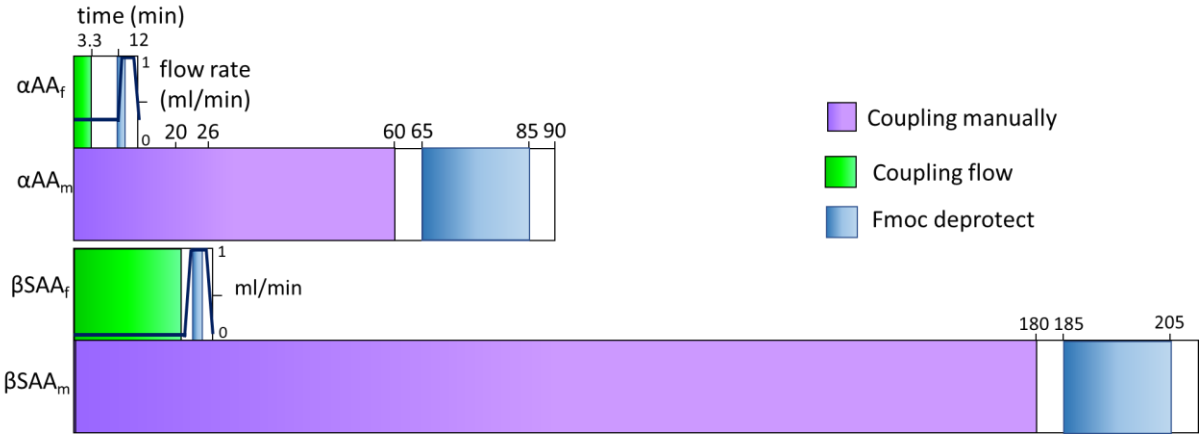


Figure 3. Comparison of one typical cycle of a manual (purple box) and flow (green box) peptide synthesis used for αAAs and modified for βSAAs. Coupling (green and purple box), deprotection (blue box) and washing (white box) steps form a complete "cycle" are depicted, all of them repeated as many times as number of the residues are coupled to the resin. Flow rates in ml/min (blue line) and their changes are indicated as function of the time.

Comparing the full coupling-deprotection-washing cycle of the manual synthesis with that of the flow chemistry we witness and benefit from the spectacular reduction of the time requested. In total, the cycle for an αAA takes 12, instead of 90 minutes, by using the αAA-protocol developed previously.[44] In this case, the actual αAA coupling requires only 3.3 minutes at a 0.3 ml/min flow rate and using an excess of 3 equivalent. In addition, Fmoc-removal is done in 1.4 minutes, using 40% piperidine in DMF. More importantly, for βSAAs the 3 hours long coupling time is reduced to 20 minutes, when flow chemistry is applied to make chimera. The newly improved protocol of 26 minutes long in total per βSAA operates at a flow rate of 0.05 ml/min only. (Time for deprotection is 1.4 min at a 1 ml/min flow rate.) In summary, the total synthesis time of the above 5-mer chimera takes as long as 10.25 hours (615 min) when competed manually, but only slightly over then one hour (84 min) when

using flow chemistry. Thus, a significant increase in efficiency (about 10 times) is witnessed, and the purity of the crude products is comparable or even superior (**Table 3**).

Table 3. The sequence and purity of the model peptides made by flow and manual synthesis

Primary sequence of the chimera (number of peptide)	**Crude product purity (%)[a]**	
	Manual	**Flow**
Ac-SG-**RibAFU**-GD-OH/NH_2[b] (**6/15**)	48	62
Ac-RG-**RibAFU**-QG-OH/NH_2[b] (**7/16**)	61	36
Ac-GW-**RibAFU**-YG-OH/NH_2 (**8/17**)	56	50
Ac-SG-**GlcAPU(Me,Bn)**-GD-OH/NH_2 (**9/18**)	93	95
Ac-RG-**GlcAPU(Me,Bn)**-QG-OH/NH_2 (**10/19**)	80	90
Ac-GW-**GlcAPU(Me,Bn)**-YG-OH/NH_2 (**11/20**)	82	87

a) The purity of the crude peptides was identified based on RP or HILIC-HPLC chromatograms
b) C-terminus is -COOH in manual and -$CONH_2$ in flow synthesis

In the case of -RibAFU- containing chimera, the purity of the crude products was similar (peptide **8**/**17**) or worse (peptide **7**/**16**), if made by flow chemistry (**Table 3**). For Ac-GW-**RibAFU**-YG-OH/NH_2 (**8/17**) oligopeptides, both manual and flow syntheses resulted in an X-sequence mistake, detected both by HPLC and MS. For the Ac-RG-**RibAFU**-QG-OH/NH_2 (**7/16**) chimera, coupling to Q was not complete during flow chemistry (61% *vs* 36% crude product purity). Thus, the coupling of the –RibAFU(ip)- moiety was not efficient enough to a large side chain/protected amino acids (like Y or Q), especially if using the "faster" flow protocol. Lower efficacy might rise from the presence of the stretched bicyclo rigid structure of the 1,2-*O*-protected ribofuranoid ring. In the case of -GlcAPU(Me,Bn)- sugar amino acid couplings are sufficiently efficient, as here the more flexible pyranoid ring is to be used, despite of the bulky benzyl side chain protecting groups on it.

Synthesis of the 16-mer penetratin and analogues

Penetratin (**21**) is a 16-mer polypeptide of high α-helical propensity even in water, a feature enhanced in lipids, membranes and in TFE.[45] Several studies focused on penetratin's secondary structure and membrane fusion ability and thus, models incorporating βSAAs (**1, 2**) influencing helical propensity is of interest. Both W6F and W14F changes were studied by ECD in RQIKI**W**FQNRRMK**W**KK penetratin and found no influence on helical propensity

in TFE/H_2O with respect to the parent molecule.[45] Besides, the Q8P replacement had no effect on penetratin internalization.[60]

In our analogues, both Trp6 alone, and Trp6-Phe7 αAA pair were replaced by a single -GlcAPU(Me,Bn)- βSAA moiety within the sequence, resulting in the W6X and W6F7X mutants, respectively. Thus, the aromatic side chain(s) of αAA(s) were replaced by the *O*-benzyl group of -GlcAPU(Me,Bn)- to probe the effect of βSAA substitution. Furthermore, Gln2 and Gln8 were changed to RibAFU(ip) and RibAFU and thus, to get more hydrophilic analogues as well as to probe their helical properties.

Table 4. Cleavage conditions and raw penetratin chimera's purity: RibAFU(ip), RibAFU and GlcAPU(Me,Bn) residues built in are highlighted.

Polypeptide/Chimera type (cleavage conditions)	**Code**	**Primary sequences**	**Crude product**	
			Purity (%)[a]	**mg[b]**
Penetratin (50%TFA, 3 hours)	**21**	RQIKIWFQNRRMKWKK	77	30
Q8**RibAFU** (50%TFA, 3 hours)	**22** **23**	RQIKIWF-**RibAFU(α/β)**-NRRMKWKK RQIKIWF-**RibAFU(ip)**-NRRMKWKK	18 47	42
Q8**RibAFU** (95%TFA, 3 hours)	**22** **23**	RQIKIWF-**RibAFU(α/β)**-NRRMKWKK RQIKIWF-**RibAFU(ip)**-NRRMKWKK	43 22	38
Q2**RibAFU** (50%TFA, 3 hours)	**24** **25**	R-**RibAFU(α/β)**-IKIWFQNRRMKWKK R-**RibAFU(ip)**-IKIWFQNRRMKWKK	44 34	47
Q2**RibAFU** (95%TFA, 3 hours)	**24** **25**	R-**RibAFU(α/β)**-IKIWFQNRRMKWKK R-**RibAFU(ip)**-IKIWFQNRRMKWKK	61 8	44
W6**GlcAPU(Me,Bn)** (50%TFA, 4.5 hours)	**26**	RQIKI[(**GlcAPU(Me,Bn**)]FQNRRMKWKK RQIK[(**GlcAPU(Me,Bn**)]FQNRRMKWKK	55 45	53
W6**GlcAPU(Me,Bn)**[c] (50%TFA, 4.5 hours)	**26**	RQIKI[(**GlcAPU(Me,Bn**)]FQNRRMKWKK	83	45
W6F7**GlcAPU(Me,Bn)** (50%TFA, 4.5 hours)	**27**	RQIKI[(**GlcAPU(Me,Bn**)]QNRRMKWKK RQIK[(**GlcAPU(Me,Bn**)]QNRRMKWKK	54 46	51
W6F7**GlcAPU(Me,Bn)**[c] (50%TFA, 4.5 hours)	**27**	RQIKI[(**GlcAPU(Me,Bn**)]QNRRMKWKK	80	48

a) Purity of the crude polypeptide and chimera were identified by RP-HPLC chromatography

b) The penetratin analogues were synthesized in 150 mg RAM-Tentagel® resin, and these data related the non-separated crude products with/without 1,2-*O*-isopropylidene protection for -RibAFU- or with/without Ile mistake for -GlcAPU(Me,Bn)- analogues

c) The protocol was changed after the GlcAPU(Me,Bn) coupling for all αAAs (RQIKI) to βSAA protocol

All penetratin analogues were synthesized by flow chemistry using the above mentioned protocols, namely for αAA (12 min), except Lys13, Gln15 and βSAA for which the longer protocol (26 min) was applied, with oxyma/DIC coupling reagents. For βSAAs the herein modified βSAA protocol was used in all cases. Firstly, both W6X and W6F7X -GlcAPU(Me,Bn)- chimera were obtained at 54 and 55% raw material purity, based on HPLC. In addition, we have identified, that Ile5 coupling to GlcAPU(Me,Bn) is problematic, as incomplete coupling resulted in characteristic side-products, namely Ile truncated chimera, were obtained. MS measurements confirmed that in the raw product the ratio of this truncated variant is as high as ~40-45% (**Table 3** and **SFigure 22**). Repeating the synthesis with a modified protocol, we have obtained the products of higher purity (80-83%) nicely purified, subsequently. In these cases, following the incorporation of βSAA all remaining αAAs were coupled using the βSAA "longer" protocol (26 min).

To cleave the GlcAPU(Me,Bn) containing chimera the previously used 50% TFA/DCM with TIS and H_2O mixture was only used (see Experiments). Furthermore, both 50% and 95% TFA ratios with TIS and H_2O were probed for RibAFU including chimera, to get both the 1,2-*O*-isopropylidene protected and the fully unprotected oligopeptides (**Table 4**). For the Q2X and Q8X penetratin analogues crude product ratios containing RibAFU(α/β) varied between 18 and 61%, while that of -RibAFU(ip) between 8 and 47% (**Table 4** and **SFigure 18, 20**), depending on the TFA ratio applied. In other words, the "cleavage cocktail's" TFA content permits to control the ratio of RibAFU(α/β)/RibAFU(ip) containing 16-mer penetratin chimera. At lower TFA% the 1,2-*O*-isopropylidene protected chimera is mostly obtained. Furthermore, the condition gives the best opportunity to discriminate between less hydrophilic RibAFU(ip) and more hydrophilic RibAFU(α/β) products, as 47% RibAFU(ip) and 18% of RibAFU(α/β)-chimera raw products were obtained (**Table 4**). On the other hand, 95% TFA gives an opposite product ratio, namely 8% RibAFU(ip) and 61% of free OH containing α/β-chimera. Therefore, if the -RibAFU- moiety is closer to the *N*- or *C*-terminus, it is easier to remove the 1,2-*O*-isopropylidene protection.

In line with our previous analysis ECD spectra were measured at different TFE/H_2O ratios to get information on the secondary structure properties of our analogues, and to make sure that the incorporation of the βSAAs did not destroy the original helical structure. For all analogues (**22-27**) we found that the expected α-helical secondary structure forms more and more, as TFE concentration increases from 0 up to 25, 50, 75 and 100 %. In the case of -RibAFU- mutants, there were no significant difference between the 1,2-*O*-isopropylidene protected and unprotected analogues. Furthermore, the bend intensities of the ECD spectra were measured for the penetratin_Q2X and found higher than in those of penetratin_Q8X mutant at the appropriate TFE concentrations (25-100% TFE, **Figure 4**). In the case of penetratin_Q2X (**24**) the intensity of the negative band at 222 nm is more intense than in the case of Q8X mutant (**22**). This indicates that the peptide Q2X has a higher ordered structure.

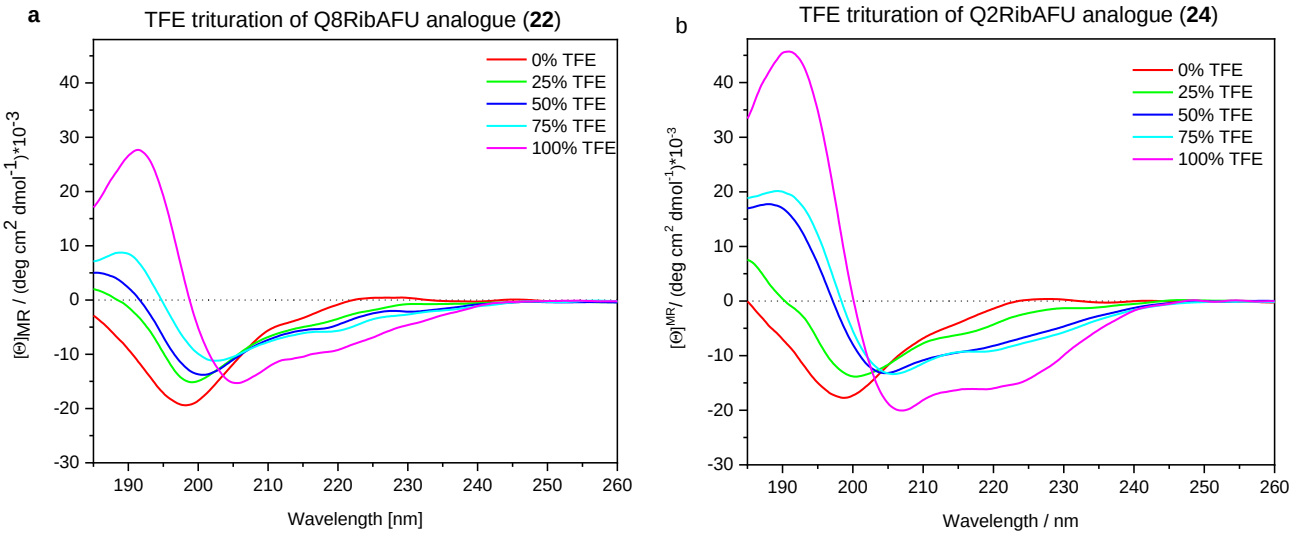


Figure 4. ECD spectra of X=RibAFU containing penetratin analogues, at different TFE/H_2O ratio. **a**) Penetratin_Q8X (**22**) **b**) Penetratin_Q2X (**24**), respectively.

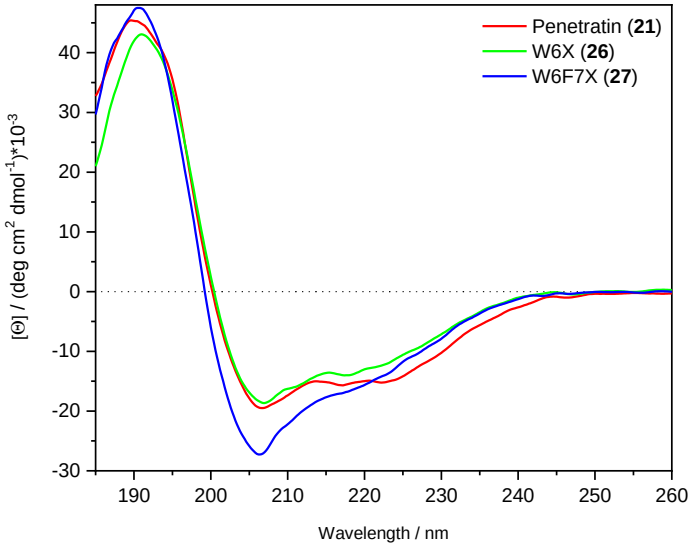


Figure 5. ECD spectra of X=GlcAPU(Me,Bn) containing penetratin analogues at 100% TFE/H_2O ratio. Penetratin (**21**), penetratin_W6X (**26**) and penetratin_W6F7X (**27**) chimera peptides.

In the case of -GlcAPU(Me,Bn)- penetratin analogues, the intensity of ECD curve is almost similar. In the case of penetratin_W6F7X the α-helical structure seems to get lower contribution to the overall structural ensemble because of the lower intensity of the negative band at 222 nm. Replacing two consecutive aromatic residues by one βSAA, penetratin_W6F7X (**27**), though retains the basic helical character of penetratin, CD spectrum shows the appearance of a larger fraction of less folded structural set as well (**Figure 5**).

Conclusions

Two βSAAs, namely Fmoc-RibAFU(ip)-OH and Fmoc-GlcAPU(Me,Bn)-OH were successfully incorporated into oligo- and polypeptides using both manual and flow-based SPPS. Short sequences containing aromatic (Y, W), large side chain protected aliphatic (R, Q) and/or tBu or OtBu protected short and polar aliphatic (S, D) α-amino acids were used to show that 50% TFA/H_2O is acidic enough to use for the final cleavage of the polypeptide from the resin but also to remove completely commercial α-amino acid's side chain protecting groups. On the other hand, 50% TFA/H_2O (with TIS) allows to control and shift product ratio of RibAFU(α/β)/RibAFU(ip)-chimera. Moreover, 50% TFA/H_2O applied for longer time (~4.5 hours) allows to completely remove the 1,2-*O*-isopropylidene protection from -RibAFU(ip)- residue, but keep intact the benzyl protection in -GlcAPU(Me,Bn)- residue, which itself can be cleaved with HF or catalytic hydrogenation. For RibAFU(α/β)-chimera in protic solvent the mutarotation determines the α/β-anomeric ratio of the product, easy to identify by 2D-NMR measurements. We used the solid phase synthetic method (SPPS) for the syntheses, because it is easy to purify due to the heterogeneous phase reaction and consists of easily automated steps. Pentameric model chimera were synthetized by flow chemistry using a new protocol worked out for βSAAs: cycle time: 26 min, flow rate: 0.05 ml/min. This method drastically reduced the time to produce the models (1.5 hours instead of 10.5 hours) and increases the coupling efficacy as raw products have the same or better purity as determined by HPLC-MS measurements.

Penetratin analogues were synthesized to investigate the incorporation and structural effects of βSAAs with respect to the original sequence. Chimera were produced at a good efficiency and purity, using flow-based SPPS method and the new 50% TFA final cleavage protocol. The ECD measurements of the analogues confirmed that βSAAs are able to maintain the helical character of the original penetratin in TFE (-RibAFU- containing Q2X and Q8X).

Moreover, one of the -GlcAPU- analogues (W6X and W6F7X) was able to form a helical structure even at a lower TFE concentration.

In summary, as function of the concentration (50 %/95 %) and type (TFA/HF) of acid used, makes possible the selective removal of the isopropylidene protecting group from -RibAFU(ip)- and/or the benzyl protection from -GlcAPU(Me,Bn)- containing chimera. They can be completed in an orthogonal manner, by either preserving or eliminating the 1,2-*O*-isopropylidene and/or *O*-benzyl protection. In this way, one can further fine-tune the hydrophilicity and aromaticity of the *Lego* type building blocks within the polypeptide chain. These recent experimental advances, with the parent hydrophobic ACPC and ACHC βAAs allow an unprecedented experimental possibility to fine-tune these biocompatible building blocks.

Experimental Session

Analytical data for all compounds (Tables, HPLC chromatograms, ECD and NMR spectra) can be found in Supporting Information, in the online version.

Materials and instrumentations

Reagents, materials and solvents were purchased from Sigma-Aldrich, Merck, Reanal, Iris Biotech and VWR. Moisture-sensitive solvents were dried on molecular sieve (3 Å).

Peptide synthesis

Model peptides were prepared manually on 2-Cl-Trt-Cl resin with Fmoc-strategy. Resin was swollen in DCM. Coupling was firstly accomplished by using Fmoc-αAA-OH (1.5 eqv. to the nominal capacity of the resin ~1.60 mmol/g to tune down to 0.23-0.56 mmol/g, see **Table 2**) dissolved in DMF, and DIEA (3.75 eqv.) added to the solution. After that, the coupling of αAAs to resin was made by reagent pairs HOBt/DIC in DMF for 1 hour while that of βSAA either -RibAFU(ip)- or -GlcAPU(Me,Bn)- was accomplished by PyBOP/DIEA in DMF for 3 hours. Finally, resin was acetylated with Ac_2O:DIEA:DMF (*v/v/v*, 1:1.2:3) for 45 mins. Resin was washed with 3 x DMF, 3 x DCM, 1 x Et_2O and dried in *vacuo* after finishing coupling and acetylation. During synthesis, the Fmoc group was removed with 2% piperidine and 2% DBU in DMF (3+17 min) and indicated by Kaiser test. The capacity of the resin was determined by UV-Vis measurement regarding to Fmoc chromophore amount (Fmoc-piperidine adduct) released by using 50% piperidine in DMF.[61]

Deprotection methods

For fully protected -RibAFU(ip)- containing peptides

Peptides (**3-5**) were cleaved from 2-Cl-Trt-Cl resins by a mixture of AcOH:MeOH:DCM (*v/v/v*, 1:1:8) for 3 hours. The resins were filtered and washed with 3 x DCM, 3 x ^{i}PrOH and 1 x Et_2O. The filtrates were removed in *vacuo*. White peptide products were precipitated by treating residues in cold Et_2O.

Ac-Ser(tBu)-Gly-RibAFU(ip)-Gly-Asp(OtBu)-OH (**3**): RP LC-MS: 10.70 min; HRMS: *m/z* calculated for $C_{29}H_{47}N_5O_{13}$ $[M+H]^+$ 674.3249, found 674.3237.
Ac-Arg(Pbf)-Gly-RibAFU(ip)-Gln(Trt)-Gly-OH (4): RP LC-MS: 15.98 min, HRMS: *m/z* calculated for $C_{57}H_{71}N_9O_{14}$ $[M+H]^+$ 1138.4919, found 1138.4905.
Ac-Gly-Trp(Boc)-RibAFU(ip)-Tyr(tBu)-Gly-OH (**5**): RP LC-MS: 15.25. min; HRMS *m/z* calculated for $C_{43}H_{56}N_6O_{13}$ $[M+H]^+$ 865.3984, found 865.3975.

For fully unprotected -RibAFU- (6-8) and protected -GlcAPU(Me,Bn)- (9-11) containing peptides

For final cleavage 50% TFA in DCM (45%), TIS (2.5%) and H_2O (2.5%) was applied on 2-Cl-Trt-Cl resin for 3 hours. The resins were filtered and washed with 3 x DCM. The filtrates were concentrated in *vacuo*, then treated with cold Et_2O to precipitate white solid peptide products.

Ac-Ser-Gly-RibAFU-Gly-Asp-OH (**6**): HILIC LC-UV-MS: 14.21 min and 14.50 min (α+β mixture), HRMS: *m/z* calculated for $C_{18}H_{27}N_5O_{13}$ $[M+H]^+$ 522.1684, found 522.1675.
Ac-Arg-Gly-RibAFU-Gln-Gly-OH (**7**): HILIC LC-UV-MS: 15.44 min, HRMS: *m/z* calculated for $C_{22}H_{37}N_9O_{11}$ $[M+H]^+$ 604.2691, found 604.2676.
Ac-Gly-Trp-RibAFU-Tyr-Gly-OH (**8**): RP LC-MS: 8.01 min, HRMS: *m/z* calculated for $C_{31}H_{36}N_6O_{11}$ $[M+H]^+$ 669.2520, found 669.2491.
Ac-Ser-Gly-GlcAPU(Me,Bn)-Gly-Asp-OH (9): RP-HPLC: 18.41 min, HRMS: *m/z* calculated for $C_{34}H_{43}N_5O_{14}$ $[M+H]^+$ 746.2833, found 746.2882.
Ac-Arg-Gly-GlcAPU(Me,Bn)-Gln-Gly-OH (10): RP-HPLC: 17.62 min, HRMS: *m/z* calculated for $C_{38}H_{53}N_9O_{12}$ $[M+H]^+$ 828.3840, found 828.3912.
Ac-Gly-Trp-GlcAPU(Me,Bn)-Tyr-Gly-OH (11): RP-HPLC: 22.11 min, HRMS: *m/z* calculated for $C_{47}H_{52}N_6O_{12}$ $[M+H]^+$ 893.3670, found 893.3726.

For O-Benzyl deprotected pentapeptides containing -GlcAPU(Me)- residues

H-Cube®: Peptide **9** (16.5 mg) was dissolved in MeOH (12 ml) and it was reduced in H-Cube® Mini reactor. The flow rate was set with an HPLC pump to 0.5 ml/min; the hydrogen pressure was set to 50 bar; the temperature to 80 °C; the cartridge containing 10% Pd/C. The resulting solution was concentrated in *vacuo* to give peptide **12** (12 mg).

Autoclave: Peptide **9** (20 mg) was dissolved in MeOH (20 ml) and put into an autoclave with 10% Pd/C (10 mg) and stirred in room temperature at 10 bar H_2 pressure during 6 hours. After that the reaction was filtered and washed with MeOH and water, then concentrated in *vacuo* resulting white solid peptide **12** (10 mg).

HF cleavage: *O*-benzyl protected cleaved peptide (**9-11**) was treated with HF gas containing 10% *p*-cresol scavenger for the HF volume in a range between -60°C and -80°C cooling with methanol/dry ice or liquid nitrogen. After the peptide solved in liquid HF the mixture was stirred at 0 °C for 1.5 hours. Then the HF was distilled from the product and the residue was treated with cold Et_2O. The mixture was filtered then the white solid was dissolved in water and lyophilized to get fully unprotected peptides (**12-14**).

Ac-Ser-Gly-GlcAPU(Me)-Gly-Asp-OH (**12**): HILIC LC-UV-MS: 14.30 min, HRMS: *m/z* calculated for $C_{20}H_{31}N_5O_{14}$ $[M+H]^+$ 566.1894, found 566,1933

Ac-Arg-Gly-GlcAPU(Me)-Gln-Gly-OH (**13**) RP-LC-UV-MS: 2.56 min, HRMS: *m/z* calculated for $C_{20}H_{31}N_5O_{14}$ $[M+H]^+$ 648.2953, found 648.2955

Ac-Gly-Trp-GlcAPU(Me)-Tyr-Gly-OH (**14**) RP-LC-UV-MS: 6.77 min, HRMS: *m/z* calculated for $C_{20}H_{31}N_5O_{14}$ $[M+H]^+$ 713.278, found 713.2812

Flow SPPS parameters

Pentapeptides (**15-20**), penetratin (**21**) and penetratin analogues (**22-27**) were synthesized by flow peptide apparatus (HPPS-4000, METALON Ltd., Hungary). Flow system consists of a conventional Jasco LC-4000 series HPLC system, except the PU-4180 HPLC pump, modified with an additional valve, allowing recirculation and regulation of solvent flow (*e.g.* cleavage mixture). ChromNAV2 software ensures the fully automated process. The autosampler injects the reagent solutions from a 1 ml sample vial, placed in the sample rack. PEEK chromatography column was used as a fixed bed reactor tube for the resin and DMF was used as solvent. 150 mg Fmoc-Rink amide TentaGel resin (0.23 mmol/g) was used. All resins were purchased from Iris Biotech GmbH. For final cleavage of peptides **15-21, 26-27** from RAM-Tentagel® 50% TFA in DCM (45%), TIS (2.5%) and H_2O (2.5%) was applied for 3 hours, except Ac-GW(GlcAPU(Me,Bn))YG-NH_2 the scavenger TIS was replaced to EDT too. Additionally, for polypeptides as penetratin analogues (**22-25**) a higher concentration of TFA (95%) with TIS (2.5%) and H_2O (2.5%) was used to achieve effectively improved removal of 1,2-*O*-isopropylidene from -RibAFU(ip)- containing peptides. The cleavage was accomplished for either 3 hours or 4.5 hours (see **Table 4**).

Fully unprotected pentapeptides containing -RibAFU- residues (15-17)

Ac-Ser-Gly-RibAFU-Gly-Asp-NH_2 (**15**): HILIC LC-UV-MS: 13.74 min and 14.12 min, (α+β mixture), HRMS: *m/z* calculated for $C_{18}H_{26}N_5O_{13}$ $[M+H]^+$ 521.1605, found 521.1832.

Ac-Arg-Gly-RibAFU-Gln-Gly-NH_2 (**16**): HILIC LC-UV-MS: 15.40 min, HRMS: *m/z* calculated for $C_{22}H_{36}N_9O_{11}$ $[M+H]^+$ 603.2613, found 603.2838.

Ac-Gly-Trp-RibAFU-Tyr-Gly-NH_2 (**17**): RP LC-MS: 7.71 min, HRMS: *m/z* calculated for $C_{31}H_{35}N_6O_{11}$ $[M+H]^+$ 668.2442, found $[M+H]^+$ 668.2654.

O-Benzyl protected pentapeptides containing -GlcAPU(Me,Bn)- residues (18-20)

Ac-Ser-Gly-GlcAPU(Me,Bn)-Gly-Asp-NH_2 (**18**): RP-HPLC: 18.51 min, MS: *m/z* calculated for $C_{34}H_{45}N_6O_{13}$ $[M+H]^+$ 745.3045, found 745.3071.

Ac-Arg-Gly-GlcAPU(Me,Bn)-Gln-Gly-NH_2 (**19**): RP-HPLC: 17.63 min, MS *m/z* calculated for $C_{38}H_{55}N_{10}O_{11}$ $[M+H]^+$ 827.4052, found 827.4066.

Ac-Gly-Trp-GlcAPU(Me,Bn)-Tyr-Gly-NH_2 (**20**): RP-HPLC: 21.62 min, MS *m/z* calculated for $C_{47}H_{54}N_7O_{11}$ $[M+H]^+$ 892.3881, found 892.3920.

Penetratin and penetratin analogues (21-27)

Penetratin (**21**): RP-HPLC: 14.99 min, HRMS: *m/z* calculated for $C_{104}H_{169}N_{35}O_{19}S$ $[M+H]^+$ 2245.3133 and $[M+5H]^{5+}$450.0705, found $[M+5H]^{5+}$ 450.0676.

Q8**RibAFU(α/β)** (**22**): RP-HPLC: 15.0 min, HRMS: *m/z* calculated for $C_{104}H_{168}N_{34}O_{21}S$ $[M+H]^+$ 2262.2922 and $[M+5H]^{5+}$453.4662, found $[M+5H]^{5+}$ 453.4639.

Q8**RibAFU(ip)** (**23**): RP-HPLC: 15.44 min, HRMS: *m/z* calculated for $C_{107}H_{172}N_{34}O_{21}S$ $[M+H]^+$ 2302.3235 and $[M+5H]^{5+}$ 461.4725, found $[M+5H]^{5+}$ 461.2712.

Q2**RibAFU(α/β)** (**24**): RP-HPLC: 15.13 min, HRMS: *m/z* calculated for $C_{104}H_{168}N_{34}O_{21}S$ $[M+H]^+$ 2262.2922 and $[M+5H]^{5+}$ 453.4662, found $[M+5H]^{5+}$ 453.4640.

Q2**RibAFU(ip)** (**25**): RP-HPLC: 15.47 min, HRMS: *m/z* calculated for $C_{107}H_{172}N_{34}O_{21}S$ $[M+H]^+$ 2302.3235 and $[M+5H]^{5+}$ 461.4725, found $[M+5H]^{5+}$ 461.4698.

W6**GlcAPU(Me,Bn) (26):** RP-HPLC: 16.5 min, HRMS: *m/z* calculated for $C_{114}H_{183}N_{34}O_{23}S$ $[M+H]^+$ 2428.3916 and $[M+5H]^{5+}$ 485.6856, found $[M+5H]^{5+}$ 485.6841

W6F7**GlcAPU(Me,Bn) (27):** RP-HPLC: 15.6 min, HRMS: *m/z* calculated for $C_{105}H_{174}N_{33}O_{22}S$ $[M+H]^+$ 2281.3232 and $[M+5H]^{5+}$ 457.2719, found $[M+5H]^{5+}$457.2698

RP-HPLC measurements

Products were measured by RP-HPLC on Aeris^{TM} 3.6 µm peptide XB-C18 100 Å, LC Column 250 x 4.6 mm with eluents 0.1% TFA in H_2O (A) and 0.08% TFA, 95% MeCN/5% H_2O (B), flow rate 0.9 ml/min and UV-detection at 220 and 280 nm. Gradient was as follow: 0 min: 5% B, 30 min: 95% B, 33 min: 95% B, 33.1 min: 5% B, 45 min: 5% B.

UHPLC-MS measurements

Products were analysed by UHPLC-MS, either RP- or HILIC- conditions. Dionex 3000 UHPLC was coupled to a Q Exactive Focus orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). ESI-MS spectra were acquired in *m/z* 200-2000 (spray voltage: 3.5 kV; sheath gas: 46 au; aux. gas: 11 au; capillary temp: 360 °C; probe heater: 406 °C). For RP LC-UV-MS, the measurements were carried out on a Waters Acquity BEH C18 column (2.1x150 mm, 1.7 µm) using different eluents such as 0.1% formic acid (FA) in H_2O (A) and 0.1% FA, 80% MeCN (B) for pentapeptides. Flow rate was 300 µl/min and 40 °C column temperature. Gradient was 0 min: 2% B, 1 min: 2% B, 17 min: 100% B, 18 min: 100% B, 22 min: 2% B and 22.1 min: 2% B. Oligopeptides having high hydrophilicity were particularly measured by HILIC LC-UV-MS on Waters Acquity BEH Amide UHPLC column (2.1x150 mm, 1.7 µm) with eluents 20 mM ammonium acetate (A) and 100% MeCN (B), flow rate 250 µl/min, UV detection at 210 and 280 nm and 40 ^{o}C column temperature. Gradient was 0 min: 90% B, 2 min: 90% B, 22 min: 40% B, 23 min: 40% B, 24 min: 90% B and 30 min: 90% B.

Preparative HPLC purification

The peptides were purified by a Jasco LC-2000Plus series preparative HPLC equipped with a binary pump and a diode array detector at 220 nm. The mobile phase consisted of 0.1% TFA in H_2O (A) and 0.1% TFA in MeCN (B) with flow rate of 3.5-5.0 ml/min. Model peptide (**5**, **8**) were purified on Phenomenex Kinetex® C-18, 100 Å, 250x10 mm, 5µm column. Gradient was applied from 20% to 80% B in 120 min for isolation of peptide **5** and from 10% to 70% B in 60 min for that of peptide **8**. Penetratin (**21**) and penetratin analogues (**22**-**27**) were isolated on Phenomenex Jupiter® C-12, 10 µm Proteo 90 Å, 250x10 mm column in the same instrument. Gradient was applied from 5% to 65% B in 120 min (**21**-**25**) or 0% to 70% in 70 min (**26**-**27**). The fractions were collected and examined by RP-HPLC. Pure products were performed by lyophilizing combined fractions.

NMR measurements

All NMR experiments were performed on Bruker Avance III 700 MHz spectrometer equipped with 5-mm z-gradient probe head operating at 298 K. Samples were dissolved in 90% H_2O, 10% D_2O and DSS/Azid (1%) with concentration 1.85 mM for peptide **8** and 0.24 mM for peptide **5**. 2D NMR measurements (^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{1}H ROESY and ^{1}H-^{1}H NOESY) were performed by using standard Bruker pulse programs. In TOCSY measurements the mixing time was 80 msec. Spectra evaluation was completed with TopSpin 4.1.1 software.

ECD measurements

ECD measurements were carried out in Jasco J-810 Spectrophotometer. Five scans were acquired with scanning speed of 50 nm/min, a cell path length of 0.1 cm, between 185 and 260 nm, at 25 ^{o}C. Concentrations of peptide samples were 0.2-0.5 mg/ml. The measurements were executed in mixtures of TFE/water with different ratios (0%, 25%, 50%, 75%, 100%). Spectra evaluation was completed with Spectra Manager program and Origin 2020b program.

Acknowledgements

This research project was supported by the European Union and the State of Hungary and financed by the European Regional Development Fund (VEKOP- 2.3.2- 16- 2017- 00014) grant of the NKFIH of the Hungarian Academy of Sciences. This work was completed in the ELTE Thematic Excellence Program (Szint+) supported by the Hungarian Ministry for Innovation and Technology. The authors gratefully acknowledge Gitta Schlosser and Adina Borbély for their help in HILIC and RP LC-UV-MS and HRMS measurements. We also wish

to thank Zoltán Bánóczi for HF cleavage support and Attila Dobó for the using of the autoclave.

References

1. Risseeuw, M.; Overhand, M.; Fleet, G. W. J.; Simone, M. I. *Amino Acids* **2013,** *45*, 613-689.
2. Hill, D. J.; Mio, M. J.; Prince, R. B.; Hughes, T. S.; Moore, J. S. *Chem. Rev.* **2001,** *101*, 3893-4011.
3. Suhara, Y.; Yamaguchi, Y.; Collins, B.; Schnaar, R. L.; Yanagishita, M.; Hildreth, J. E. K.; Shimada, I.; Ichikawa, Y. *Bioorg. Med.Chem.* **2002,** *10*, 1999-2013.
4. Gruner, S. A. W.; Truffault, V.; Voll, G.; Locardi, E.; Stockle, M.; Kessler, H. *Chem. Eur. J.* **2002,** *8*, 4366-4376.
5. Chandrasekhar, S.; Reddy, M. S.; Jagadeesh, B.; Prabhakar, A.; Rao, M.; Jagannadh, B. *J. Am. Chem. Soc.* **2004,** *126*, 13586-13587.
6. von Roedern, E. G.; Lohof, E.; Hessler, G.; Hoffmann, M.; Kessler, H. *J. Am. Chem. Soc.* **1996,** *118*, 10156-10167.
7. Fujimura, F.; Hirata, T.; Morita, T.; Kimura, S.; Horikawa, Y.; Sugiyama, J. *Biomacromolecules* **2006,** *7*, 2394-2400.
8. Ishihara, Y.; Kimura, S. *Biopolymers* **2013,** *100*, 141-147.
9. Song, Z.; He, X.-P.; Chen, G.-R.; Xie, J. *Synthesis* **2011,** *2011*, 2761-2766.
10. Ménand, M.; Blais, J.-C.; Hamon, L.; Valéry, J.-M.; Xie, J. *J. Org. Chem.* **2005,** *70*, 4423-4430.
11. Siriwardena, A.; Pulukuri, K. K.; Kandiyal, P. S.; Roy, S.; Bande, O.; Ghosh, S.; Garcia Fernández, J. M.; Ariel Martin, F.; Ghigo, J.-M.; Beloin, C.; Ito, K.; Woods, R. J.; Ampapathi, R. S.; Chakraborty, T. K. *Angew. Chem. Int. Ed.* **2013,** *52*, 10221-10226.
12. Rinaldi, S. *Molecules* **2020,** *25*, 3276.
13. Blanco, A.; Blanco, G. In *Medical Biochemistry;* Blanco, A.; Blanco, G. Eds.; Academic Press, 2017; pp. 73-97.
14. Gervay-Hague, J.; Weathers, J. T. M. *J. Carbohydr. Chem.* **2002,** *21*, 867-910.
15. Williamson, A. R.; Zamenhof, S. *J. Biol. Chem.* **1963,** *238*, 2255-2258.
16. Nazarenko, E. L.; Crawford, R. J.; Ivanova, E. P. *Marine Drugs* **2011,** *9*.
17. Coutsogeorgopoulos, C.; Bloch, A.; Watanabe, K. A.; Fox, J. J. *J. Med. Chem.* **1975,** *18*, 771-776.
18. Suhara, Y.; Kurihara, M.; Kittaka, A.; Ichikawa, Y. *Tetrahedron* **2006,** *62*, 8207-8217.
19. Nagy, A.; Csordás, B.; Zsoldos-Mády, V.; Pintér, I.; Farkas, V.; Perczel, A. *Amino Acids* **2017,** *49*, 223-240.
20. Goldschmidt Gőz, V.; Pintér, I.; Harmat, V.; Perczel, A. *Eur. J. Org. Chem.* **2018**, 355-361.
21. Csordás, B.; Nagy, A.; Harmat, V.; Zsoldos-Mády, V.; Leveles, I.; Pintér, I.; Farkas, V.; Perczel, A. *Amino Acids* **2016,** *48*, 2619-2633.
22. Heyns, K.; Kiessling, G.; Lindenberg, W.; Paulsen, H.; Webster, M. E. *Chem. Ber.* **1959,** *92*, 2435 - 2438.
23. Torres, E.; Gorrea, E.; Burusco, K. K.; Da Silva, E.; Nolis, P.; Rua, F.; Boussert, S.; Diez-Perez, I.; Dannenberg, S.; Izquierdo, S.; Giralt, E.; Jaime, C.; Branchadell, V.; Ortuno, R. M. *Org. Biomol. Chem.* **2010,** *8*, 564-575.
24. Appella, D. H.; Christianson, L. A.; Klein, D. A.; Richards, M. R.; Powell, D. R.; Gellman, S. H. *J. Am. Chem. Soc.* **1999,** *121*, 7574-7581.
25. Hetényi, A.; Mándity, I. M.; Martinek, T. A.; Tóth, G. K.; Fülöp, F. *J. Am. Chem. Soc.* **2005,** *127*, 547-553.
26. Gellman, S. H. *Acc. Chem. Res.* **1998,** *31*, 173-180.
27. Seebach, D.; Matthews, J. L. *Chem.Commun.* **1997**, 2015-2022.
28. Martinek, T. A.; Fülöp, F. *Chem. Soc. Rev.* **2012,** *41*, 687-702.
29. Hager, M. V.; Clydesdale, L.; Gellman, S. H.; Sexton, P. M.; Wootten, D. *Biochem. Pharma.* **2017,** *136*, 99-108.
30. Fortuna, P.; Twarda-Clapa, A.; Skalniak, L.; Ożga, K.; Holak, T. A.; Berlicki, Ł. *Eur. J. Med. Chem.* **2020,** *208*, 112814.
31. Johnson, L. M.; Barrick, S.; Hager, M. V.; McFedries, A.; Homan, E. A.; Rabaglia, M. E.; Keller, M. P.; Attie, A. D.; Saghatelian, A.; Bisello, A.; Gellman, S. H. *J. Am. Chem. Soc* **2014,** *136*, 12848-12851.

32. Franconetti, A.; Jatunov, S.; Borrachero, P.; Gómez-Guillén, M.; Cabrera-Escribano, F. *Org. Biomol. Chem.* **2013,** *11*, 676-686.
33. Reist, E. J.; Spencer, R. R.; Calkins, D. F.; Baker, B. R.; Goodman, L. *J. Org. Chem.* **1965,** *30*, 2312-2317.
34. Vera-Ayoso, Y.; Borrachero, P.; Cabrera-Escribano, F.; Gómez-Guillén, M. *Tetrahedron: Asymmetry* **2005,** *16*, 889-897.
35. Taillefumier, C.; Lakhrissi, Y.; Lakhrissi, M.; Chapleur, Y. *Tetrahedron: Asymmetry* **2002,** *13*, 1707-1711.
36. Soengas, R. G. *Synlett* **2010,** *2010*, 2549-2552.
37. Gasch, C.; Merino-Montiel, P.; López, Ó.; Fernández-Bolaños, J. G.; Fuentes, J. *Tetrahedron* **2010,** *66*, 9964-9973.
38. Zhang, K.; Wang, J.; Sun, Z.; Nguyen, D.-H.; Schweizer, F. *Synlett* **2007,** *2007*, 0239-0242.
39. Watterson, M. P.; Pickering, L.; Smith, M. D.; Hudson, S. J.; Marsh, P. R.; Mordaunt, J. E.; Watkin, D. J.; Newman, C. J.; Fleet, G. W. J. *Tetrahedron: Asymmetry* **1999,** *10*, 1855-1859.
40. Tuwalska, D.; Sienkiewicz, J.; Liberek, B. *Carbohydrate Research* **2008,** *343*, 1142-1152.
41. Hoffmann, M.; Burkhart, F.; Hessler, G.; Kessler, H. *Helv. Chim. Acta* **1996,** *79*, 1519-1532.
42. Nagy, A.; Goldschmidt Gőz, V.; Pintér, I.; Farkas, V.; Perczel, A. *Amino Acids* **2019,** *51*, 669-678.
43. Duong, K. H. Y.; Goldschmidt Gőz, V.; Pintér, I.; Perczel, A. *Amino Acids* **2021,** *53*, 281-294.
44. Farkas, V.; Ferentzi, K.; Horváti, K.; Perczel, A. *Org. Process Res. Dev.* **2021,** *25*, 182-191.
45. Czajlik, A.; Meskó, E.; Penke, B.; Perczel, A. *J. Pept. Sci.* **2002,** *8*, 151-171.
46. Garibotto, F. M.; Garro, A. D.; Rodríguez, A. M.; Raimondi, M.; Zacchino, S. A.; Perczel, A.; Somlai, C.; Penke, B.; Enriz, R. D. *Eur. J. Med. Chem.* **2011,** *46*, 370-377.
47. Garro, A. D.; Olivella, M. S.; Bombasaro, J. A.; Lima, B.; Tapia, A.; Feresin, G.; Perczel, A.; Somlai, C.; Penke, B.; López Cascales, J.; Rodríguez, A. M.; Enriz, R. D. *Chem. Biol. Drug Design* **2013,** *82*, 167-177.
48. Letoha, T.; Gaál, S.; Somlai, C.; Czajlik, A.; Perczel, A.; Penke, B. *J. Mol. Recognit.* **2003,** *16*, 272-279.
49. Parravicini, O.; Somlai, C.; Andujar, S. A.; Garro, A. D.; Lima, B.; Tapia, A.; Feresin, G.; Perczel, A.; Tóth, G.; Cascales, J. L.; Rodríguez, A. M.; Enriz, R. D. *Archiv der Pharmazie* **2016,** *349*, 242-251.
50. Jallouk, A. P.; Palekar, R. U.; Pan, H.; Schlesinger, P. H.; Wickline, S. A. In *Adv. Protein Chem. Struct. Biol.;* Donev, R. Ed.; Academic Press, 2015; pp. 57-91.
51. Zhu, X.; Jin, K.; Huang, Y.; Pang, Z. In *Brain Targeted Drug Delivery System;* Gao, H.; Gao, X. Eds.; Academic Press, 2019; pp. 159-183.
52. Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. *J Mol Biol* **1990,** *215*, 403-10.
53. Shi, M.; Foo, S. Y.; Tan, S. M.; Mitchell, E. P.; Law, S. K.; Lescar, J. *J Biol Chem* **2007,** *282*, 30198-206.
54. Kobayashi, N., Kigawa, T., Koshiba, S., Tochio, N., Inoue, M., Yokoyama, S.: PDB Database, 2003.
55. Politou, A. S.; Millevoi, S.; Gautel, M.; Kolmerer, B.; Pastore, A. *J. Mol. Biol.* **1998,** *276*, 189-202.
56. Oyen, D.; Torres, J. L.; Wille-Reece, U.; Ockenhouse, C. F.; Emerling, D.; Glanville, J.; Volkmuth, W.; Flores-Garcia, Y.; Zavala, F.; Ward, A. B.; King, C. R.; Wilson, I. A. *PNAS* **2017,** *114*, E10438.
57. Scally, S. W.; McLeod, B.; Bosch, A.; Miura, K.; Liang, Q.; Carroll, S.; Reponen, S.; Nguyen, N.; Giladi, E.; Rämisch, S.; Yusibov, V.; Bradley, A.; Lemiale, F.; Schief, W. R.; Emerling, D.; Kellam, P.; King, C. R.; Julien, J.-P. *Nat. Commun.* **2017,** *8*, 1568.
58. Pei, Z.; Li, X.; Longenecker, K.; von Geldern, T. W.; Wiedeman, P. E.; Lubben, T. H.; Zinker, B. A.; Stewart, K.; Ballaron, S. J.; Stashko, M. A.; Mika, A. K.; Beno, D. W. A.; Long, M.; Wells, H.; Kempf-Grote, A. J.; Madar, D. J.; McDermott, T. S.; Bhagavatula, L.; Fickes, M. G.; Pireh, D.; Solomon, L. R.; Lake, M. R.; Edalji, R.; Fry, E. H.; Sham, H. L.; Trevillyan, J. M. *J. Med. Chem.* **2006,** *49*, 3520-3535.

59. Houben, H. J.; Weyl, T. *Synthesis of Peptides and Peptidomimetics*, 4th Edition ed.; Georg Thieme Verlag: Stuttgart and New York, 2002; Vol. E 22a-e.
60. Derossi, D.; Calvet, S.; Trembleau, A.; Brunissen, A.; Chassaing, G.; Prochiantz, A. *J Biol Chem* **1996,** *271*, 18188-93.
61. Chan, W.; White, P. *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*; Oxford University Press: Oxford, 1999.