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LETTER

γ-AApeptides: design, synthesis and evaluation†

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A new class of peptide mimics termed "γ-AApeptides" has been described. The design and synthesis of γ-AApeptides, and potential bioactivities towards p53/MDM2 interaction were demonstrated. γ-AApeptides were also found to be highly resistant to proteolysis. The development of sequence-specific γ-AApeptides may lead to a family of peptidomimetics with a new framework for drug discovery or peptide/protein mimicry.

Sequence-specific peptidomimetic oligomers with a variety of frameworks, structures and functions are increasingly important in chemical biology and biomedical sciences.[1,2] In the last decade there have been extensive efforts to develop biomimetic oligomers, such as β-peptides,[3–5] peptoids,[6] α-aminoxy-peptides,[7] α/β-peptides,[8,9] azapeptides,[10,11] and phenylene ethynylenes,[12] *etc*. These peptidomimetics have many advantages over conventional peptides including resistance to enzymatic hydrolysis and improved bioavailability, due to the presence of their unnatural backbones.[13] The area of research has led to some important functions and applications of peptidomimetics ranging from drug discovery to protein surface mimicry.[14,15] Nonetheless, the development of peptidomimetics is far less straightforward than expected, limited by the availability of backbones. A variety of backbones are needed to generate different classes of peptide mimics with discrete structures and functions.[2] Novel peptide mimics with new frameworks are crucial in order to further understand protein folding and functional properties, to generate a new chemically diverse library so as to identify novel therapeutic agents, and to further facilitate the application of peptidomimetics in chemical biology.[2,9]

In an effort to search for new class of peptide mimics to facilitate drug discovery and protein surface mimicry, we herein describe a new family of sequence-specific peptide mimics derived from γ-chiral PNAs.[16–18] They are termed "γ-AApeptides" because they are comprised of γ-substituted-*N*-acylated-*N*-aminoethyl amino acids (Fig. 1). Such γ-chiral PNA backbones were chosen because of their superior resistance to proteolysis and their potential for peptide mimicry.

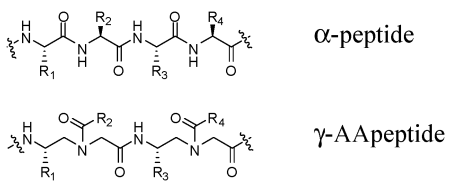

Fig. 1 Structures of an α-peptide and the corresponding γ-AApeptide.

The repeating unit of γ-AApeptide is comparable to a di-α-peptide residue. In such a unit, there are two side chains; one is from an α-amino acid side chain, while the other comes from a tertiary amide. In γ-AApeptides, nitrogen atoms on the backbone are either forming secondary or tertiary amide bonds. γ-AApeptide side chains on the γ position of the backbone are derived from α-amino acids. Compared with conventional α-peptides, γ-AApeptides are able to project the same number of side functional groups on the backbone of same length. Therefore, γ-AApeptides were presumably able to mimic peptide structures and reproduce their functions, and generate a novel chemically diverse library for drug discovery. One should be aware that although the primary structures of γ-AApeptides can in principal mimic those of α-peptides (side chains of γ-AApeptides can perfectly overlap with those of α-peptides), direct inter-conversion of sequences between γ-AApeptides and α-peptides may not present same functions or bioactivities. This is because γ-AApeptide and peptide backbones are different despite some similarities, therefore their hydrogen bonding properties and conformational flexibilities are not identical (γ-AApeptides tend to be more flexible). Such distinctions will result in different conformational structures, which lead to possibly different functional behavior.

Bioactivity is one of the most important functions expected for peptidomimetics. As the proof of concept, initially we chose p53/MDM2 interaction as the modeling system to investigate if certain γ-AApeptides can disrupt this protein–protein interaction using an ELISA assay. This protein–protein interaction was chosen because it has been a testing basis for the design strategy of sequence-specific peptidomimetics.[19] Peptidomimetics with different scaffolds such as β-peptides,[20] peptoids,[21,22] and *N*-acylpolyamine[23] have been reported to disrupt p53/MDM2 interactions. Hence, any activity shown for γ-AApeptides to inhibit this interaction would be important for evaluating their

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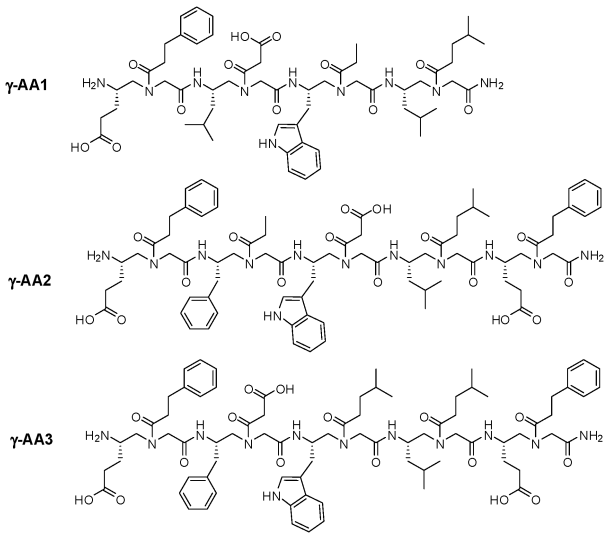

Fig. 2 γ-AApeptide sequences designed for p53/MDM2 disruption.

capability to mimic peptides and to demonstrate their potential application in chemical biology. Therefore, we designed several γ-AApeptide sequences (Fig. 2) bearing side functional groups of phenylalanine, tryptophan, and leucine, which are critical for disruption of p53/MDM2 interaction.[19]

We initially attempted to directly synthesize γ-AApeptides on a solid phase through a submonomer strategy by building the sequence step by step (Fig. 3). Unfortunately, overalkylation was constantly observed during the reductive amination step. A few coupling cycles yielded a mixture of unidentifiable products on HPLC after cleavage from the solid phase.

We then carried out the synthesis using a "monomer building block" strategy (Fig. 4), the same protocol adopted from the standard solid phase synthesis of conventional peptides.

In this route, γ-AApeptide building blocks were prepared and used for solid phase synthesis. Briefly, Fmoc-amino aldehydes[18,24] reacted with glycine benzyl ester through reductive amination; the products were then acylated with carboxylic acids. Subsequent hydrogenation provided desired γ-AApeptide building blocks, which were assembled on a Knorr resin using Fmoc chemistry to produce the γ-AApeptides. Preparation of **3** under different conditions was investigated. Oxohydroxybenzotriazole/DIC was found to be efficient for the coupling

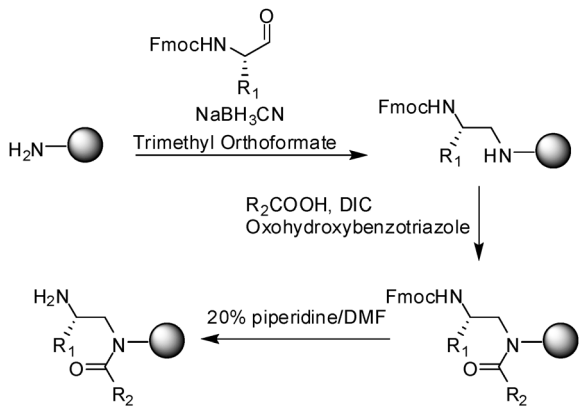

Fig. 3 Initial unsuccessful attempt to synthesize γ-AApeptides.

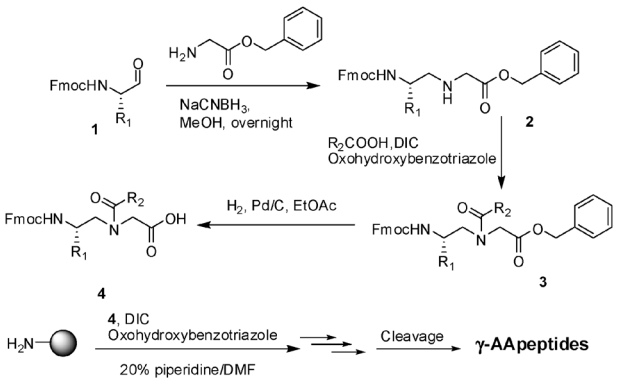

Fig. 4 Synthesis of γ-AApeptide building block

with most carboxylic acids, while the use of other activation agents such as PyBOP, HBTU/HOBt, DIC/HOBt provided the desired product **3** when **2** was coupled with a few acids, but in most cases the yields were poor. Solid phase synthesis of γ-AApeptides by assembling γ-AApeptide building blocks **4** was problem-free and highly efficient. These sequences were prepared over 80% yield in crude and eluted by HPLC with more than 95% purity (Fig. 5). Three γ-AApeptides were described here for demonstration. Due to the freedom of using almost countless carboxylic acids, the derivatization of γ-AApeptides is virtually unlimited, which allows the generation of a γ-AApeptide library relatively simply and with much more diversity than regular peptides. Such features will greatly expand their potential applications in drug discovery in the future.

The above sequences were then investigated for their capability to disrupt p53/MDM2 protein–protein interactions by the ELISA assay (Table 1). A previously reported p53-derived peptide[25] is included for comparison.

ELISA results show that γ-AA**3**, with an IC_{50} of 50 μM, is an effective inhibitor of p53/MDM2 interaction. It is only a few fold less potent than α-peptides, and comparable to some peptoids or β-peptides reported previously.[21,26] This result successfully demonstrated the functions of γ-AApeptide as novel peptide mimics. The selectivity of γ-AApeptides is also excellent. Different selected sequences are giving different activities for p53/MDM2 inhibition. γ-AA**1** has the least activity, while γ-AA**3** is the best inhibitor among three sequences. This suggests that γ-AApeptides do not interact with protein

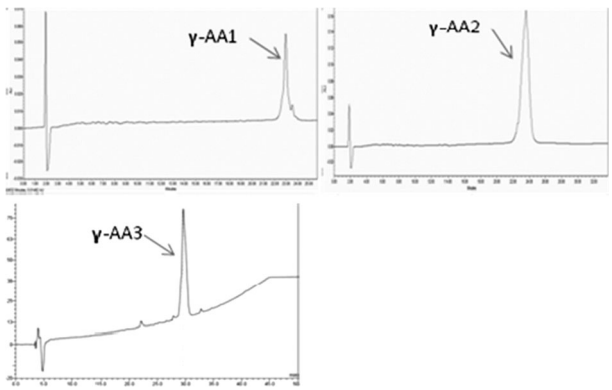

Fig. 5 HPLC traces of γ-AApeptides after purification.

Table 1 ELISA results of γ-AApeptides for the disruption of p53/MDM2

γ-AApeptides	$IC_{50}/\mu M$
γ-AA1	>400
γ-AA2	120 ± 15
γ-AA3	50 ± 8
p53-derived peptide (Ac-QETFSDLWKLLP)	8.7[25]

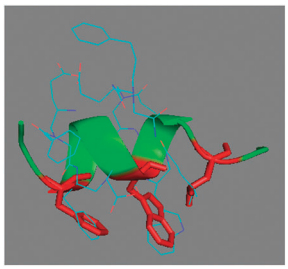

Fig. 6 Energy minimized structure (MM2) of γ-AApeptide **3** is superimposed with the p53 17-29 helical domain (blue colored). γ-AApeptide **3** is shown as the wire frame presentation. Three critical residues (Phe19, Trp23, and Leu 26) in p53 responsible for binding to MDM2 are red colored.

randomly. Computer modeling (Fig. 6) indeed shows that the side groups of an energy-minimized structure of γ-AA**3** are able to overlap with Phe, Trp, and Leu of the p53 helical domain that interacts with MDM2. Although a detailed structure–activity relationship (SAR) will require the test of more derivatives, and it is beyond the scope of this report, much more potent γ-AApeptide inhibitors are expected with the introduction of halogen atoms onto the indole group of the Trp residue[21,27] and/or bulky and charged groups,[19] and with the assistance of *in silico* computer modeling and screening.

Another important feature of sequence-specific peptidomimetics is their superior resistance to enzymatic hydrolysis, owing to their artificial backbones, which is a key advantage over regular peptides. To determine the stability of γ-AApeptides against enzyme degradation, the typical sequence **3** was incubated, respectively, with chymotrypsin, thermolysin, and pronase (0.1 mg ml^{-1}) in a 100 mM pH 7.8 ammonium bicarbonate buffer for 24 h. HPLC was used to analyze the reaction mixtures by comparing their retention time, integration and molecular weight to those of the starting material. The results (see ESI†) show that AApeptides are potentially highly resistant to proteolysis. None of the above proteinases is able to hydrolyze **3** after 24 h incubation.

In conclusion, we have designed a new family of peptide mimics—γ-AApeptides—and demonstrated their efficient synthesis on the solid phase using a monomer approach based on γ-substituted *N*-acylated-*N*-Fmocamino ethyl amino acid building blocks. Diversifying γ-AApeptides by introducing a wide variety of side chains onto γ-AApeptide backbones is straightforward and easy considering the availability of numerous carboxylic acids. Furthermore, γ-AApeptides are found to be highly resistant to enzymatic degradation. Further characterization and development of sequence-specific γ-AApeptides will enrich the current peptidomimetic family and expand their potential applications in drug discovery and chemical biology by introducing new frameworks and structures. Obtaining structural requirements for γ-AApeptides to form predicable structures using Circular Dichroism (CD), 1D/2D NMR, and X-ray crystallography, and design of novel γ-AApeptide sequences as better inhibitors/effectors for p53/MDM2 and other proteins/nucleic acids/membrane are currently ongoing.

Experimental

General experimental methods

All the reagents and solvents were used as received without further purification. γ-AApeptide sequences were prepared on a Knorr resin in peptide synthesis vessels, analyzed and purified on an analytical and a preparative HPLC, respectively, and then lyophilized. Molecular weights of γ-AApeptides were identified on a MALDI-TOF mass spectrometer.

Typical synthesis of 2

A glycine benzyl ester hydrochloride in 15 ml methanol in a 100 ml round bottom flask was added to 1.1 equiv. of triethylamine and stirred at 0 °C for 15 min. A stoichiometric amount of a Fmoc protected amino acid aldehyde[18,24] was added and the solution mixture was stirred for another 30 min. A catalytic amount of acetic acid was then added followed by 2 equiv. of $NaBH_3CN$. The solution was allowed to stir at 0 °C for 1 h and continued at room temperature overnight. The solvent was evaporated and 100 ml ethyl acetate and 100 ml saturated sodium bicarbonate solution were added to the residue. The organic layer was separated and washed with 100 ml brine, dried over anhydrous sodium sulfate, and removed *in vacuo*. Flash chromatography using ethyl acetate/hexane 1 : 1 gave **2** (R_f = 0.2) as a colorless oil.

Typical synthesis of 3

Compound **2**, 1.2 equiv. of DIC, oxohydroxybenzotriazole, and R_2COOH were stirred in 20 ml DMF overnight. The solution was then partitioned in 100 ml ethyl acetate and 100 ml water. The organic layer was separated and washed with water (3 × 100 ml) and brine (2 × 100 ml), dried over anhydrous sodium sulfate, and then concentrated *in vacuo*. Flash chromatography using ethyl acetate/hexane 1 : 3 gave **3** (R_f = 0.1) as a colorless oil.

Typical synthesis of 4

3 in 20 ml ethyl acetate was added to 10% Pd/C and hydrogenated at atmospheric pressure and room temperature overnight. The solution was evaporated and the residue was purified by flash chromatography, 5–7% $MeOH/CH_2Cl_2$, to give **4** (R_f = 0.2 in 7% $MeOH/CH_2Cl_2$) as a white foam solid.

Synthesis of γ-AApeptides

γ-AApeptides were prepared on the Knorr resin in peptide synthesis vessels on a shaker following the standard Fmoc chemistry of the solid phase peptide synthesis protocol. Each coupling cycle included an Fmoc deprotection using 20% piperidine in DMF, and 4 h coupling of 1.5 equiv. of γ-AApeptide building blocks onto the resin in the presence of 2 equiv. of DIC/oxohydroxybenzotriazole in DMF.

After desired sequences were assembled, they were transferred into a 4 ml vial and cleaved from the solid support in 74 : 24 : 2 TFA/CH_2Cl_2/triisopropylsilane for 2 h. Then the solvent was evaporated and the residues were purified by HPLC. The desired fractions were collected and lyophilized.

ELISA assay

GST-MDM2-1-150 and full-length His6-p53 were expressed in *E. coli* and affinity purified under non-denaturing conditions. ELISA plates were incubated with 2.5 μg ml^{-1} His6-p53 in phosphate buffered saline (PBS) for 16 h. After washing with PBS + 0.1% Tween 20 (PBST), the plates were blocked with PBS + 5% non-fat dry milk + 0.1% Tween 20 (PBSMT) for 0.5 h. GST-HDM2 was mixed with γ-AApeptides in PBSMT + 10% glycerol + 10 mM DTT and added to the wells. The plates were washed with PBST after incubating for 1 h at room temperature, incubated with MDM2 antibody 4B2 in PBSMT for 1 h, followed by washing and incubation with HRP–rabbit-anti-mouse Ig antibody for 1 h. The plates were developed by incubation with a TMB peroxidase substrate (KPL) and measured by absorbance at 450 nm.

Acknowledgements

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