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ORIGINAL PAPER

An Original Strategy for Gln Containing Peptide Synthesis Using SPPS and Glu(OH)-1-OAll

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Abstract Using multiple peptide synthesis in parallel, a series of 24 compounds analogues of tripeptide sequence Z-Leu-Phe-Gln-H, modified by imidazole moiety were synthesized. An effective and simple scheme for including imidazole heterocycle to C- and/or N-terminus of Gln residue was created by means of allyl group as α-COOH protecting group for Fmoc-Glu. The approach using Fmoc-Glu-1-OAll as a first amino acid linked to the resin could be useful for the synthesis of a large number of amino acids and/or heterocyclic moieties including compounds. Based on the preliminary biological trials we could conclude that the presence of imidazole heterocycle affect positively the antiviral activity against *Coxsackieviruses B1* and *Poliovirus type 1*.

Keywords Gln containing peptides · Allyl protecting group · Peptidomimetics · 3CP inhibitors

Introduction

Despite the large progress in peptide chemistry due to solid phase peptide synthesis (SPPS) discovered by Merrifield (1963), the synthesis of each new peptide sequence represents a major challenge and is often unpredictable, especially when obtaining peptides with high molecular weight and difficult sequences. The creation of new synthetic schemes for synthesis of peptides is provoked both by the discovery of new methods for condensation and new protective groups (alone or in combination with already known ones) as well as the possibilities to apply various strategies of realizing a peptide sequence using known condensation methods.

A lot of experiments connected with compounds possessing inhibition activity against the cysteine-containing human rhinovirus 3C protease (3CP) revealed that some simple changes in the 3CP substrate H-His-Leu-Phe-Gln-Gly-Pro-OH could transform it into low molecular strong inhibitor Z-Leu-Phe-Gln-Michael acceptor (Fig. 1) (Dragovich et al. 1998a, b).

A series of similar inhibitors have been synthesized and described in the literature in order to reveal the role of different parts of the molecule on the antiviral activity (Dragovich et al. 1998a, b; Dragovich 2001; Dragovich and Prins 2003; Wang 1999).

It was proven that Leu residue at P_3 position is not necessary for the substrate-enzyme interaction (Dragovich et al. 1999). Therefore, a wide variety of substitutes are tolerated at this position. Further studies showed that aldehyde group as a Michael acceptor is not acceptable because of their toxicity, lack of selectivity and stability (Dragovich et al. 2000).

We were challenged to synthesize a series of mimetics of hexapeptide H-His-Leu-Phe-Gln-Gly-Pro-OH containing Gln, with potential antiviral activity, by means of specifically protected Glu(OH)-1-OAll residue and rink amide resin using SPPS. Herein, we report an original strategy for synthesis of these compounds as well as some preliminary antiviral tests.

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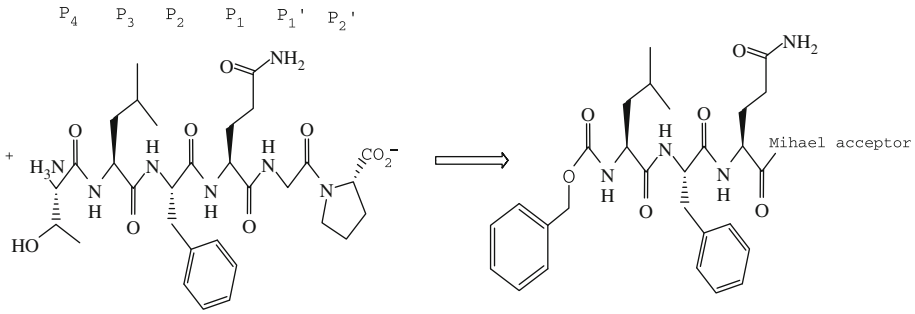


Fig. 1 Transformation model of 3CP substrate into its inhibitor

Materials and Methods

The purity of the newly synthesized compounds were monitored by the HPLC Perkin-Elmer Series 410 LC BioPump, Diode Array detector LC 235, Spherisorb 5 ODS 250 mm × 4.6 mm column, isocratic elution with 50% MeCN/50% 0.02 M KH_2PO_4:K_2HPO_4, pH = 7, flow rate 1 ml/min, $\lambda = 220$ nm.

The mass specters were recorded on the Micromas Platform LC, cone voltage 70, heat 70°C and positive or negative charge.

All syntheses were performed by multiple-peptide syntheses in parallel using SPPS, Fmoc-strategy, Rink Amide Resin (HCPam 02-1, 1% DVSB, 100–200 mesh). 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-Hydroxybenzotriazole/*N*,*N*-Diisopropylethylamine (HBTU/1-HOBt/DIPEA) were used as coupling reagents. Three fold excess of the Fmoc-amino acid compared to the resin was used.

A $Pd(PPh_3)_4$-Tetracis (3 equiv.)/$CHCl_3$-AcOH-*N*-methylmorpholine (37:2:1) (15 ml per gram resin) under argon stream was used for the 1-allyl group deblocking.

The imidazole condensation was performed by 3 equiv. of imidazole to 1 equiv. resin and O-(6-Chloro-1-hydrocibenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TCTU)/1-HOBt/DIEA (1:1.1:1.1 fold excess compared to the resin).

The coupling of 4-imidazole acetic acid was made by means of TCTU/1-HOBt/DIPEA.

The final peptides were obtained by treatment with a mixture of 2.5 ml of 95% TFA (2.4 ml), 2.5% H_2O (0.06 ml) and 2.5% Triisopropylsilane (TIS) (0.06 ml) for 2.5 h.

Chemical Part

A: Procedure for Attachment of Fmoc-Glu(OH)-OAll to Rink Amide Resin

The Rink amide resin was pre-swollen with dichloromethane (DCM) for 1 h and then washed thoroughly with *N*,*N*-dimethylformamide (DMF). Separately 4 equiv. excess compared to the resin of Fmoc-Glu(OH)-OAll dissolved in DCM and diisopropylcarbodiimide (DIC) (fivefold excess compared to the resin) were mixed and the resulting solution was stirred for 20 min at 0°C. Further the solvent was evaporated under vacuum. The obtained oil was dissolved in DMF and added to the resin followed by 4-*N*,*N*-dimethylaminopyridine (DMAP) (0.1 equiv. compared to the resin). 2.5 h later the solvent was removed under vacuum and the resin was washed with 3× DMF and 3× DCM, subsequently. A standard Kaiser test was made. The negative Kaiser test means that the reaction of condensation is successfully done.

B: General Procedure for Coupling of Amino Acids Using HBTU (TCTU)/DIPEA Method

The Fmoc-amino acid (4 equiv. relative to resin loading), HBTU (TCTU) (4 equiv. relative to resin loading) and diisopropylethylamine (DIPEA) (4 equiv. relative to resin loading) were dissolved in DMF. The reaction mixture was stirred for 10 min and added to the peptide-resin solution in DMF previously deprotected as regards the Fmoc N-terminal function. The reaction mixture was stirred for 2 h. The Kaiser test was made at the end of the reaction time. The negative Kaiser test means that the reaction of condensation is successfully done.

C: Preparing of Peptide-Resin for Cleavage and Removing the Peptide from the Resin

The obtained peptide-resin was washed by 3× DMF; 3× DCM; 3× i-propanol and 3× diethyl ether to shrink the resin. The peptide-resin was then dried under high vacuum for 4 h over NaOH. The dry peptide-resin was placed in a flask and TFA/TIS/H_2O (95:2.5:2.5) were added for 5 h. At the end of the reaction time the obtained peptide was collected by filtration and the removed resin was washed with 3× TFA and 3× DCM, subsequently. The obtained filtrates were collected and evaporated to dryness.

D: Condensation of imAc and Imidazole

ImAc or imidazole (3 equiv. to the peptide-resin) dissolved in DMF and TCTU/1-HOBt/DIPEA (1:1.1:1.1 equiv.

excess compared to the resin) were added. The reaction mixture was stirred for 5 h.

E: Allyl Group Deblocking

$Pd(PPh_3)_4$ (threefold excess to the peptide-resin) dissolved in a mixture of $CHCl_3$/AcOH/*N*-methylmorpholine (37:2:1) (15 ml/g resin) under argon atmosphere was added to the fully dried resin. The reaction mixture was stirred for 4.5 h under argon and at the end of the reaction time the resin was washed with 0.5% DIPEA in DMF and 0.5% sodium diethyldithiocarbamate in DMF for catalyst removal.

Biological Trials

The CV-B1 and LSc-2ab viruses used for our biological trials were kindly placed at our disposal by The Stephen Angeloff Institute of Microbiology at the Bulgarian Academy of Sciences. All procedures for virus and FL cells cultivation were performed according to the literature (Rada and Zavada 1962; Galabov and Galabov 1980; Galabov and Iossifova 1996; Galabov and Nikolaeva 1998; Ivanova and Milkova 1997).

The agar-diffusion plaque-inhibition test with cylinders was used for the initial screening of antiviral activity and was performed as previously described (Rada and Zavada 1962; Galabov and Galabov 1980; Galabov and Iossifova 1996; Galabov and Nikolaeva 1998; Ivanova and Milkova 1997). The presented results were obtained in two independent experiments with three replications in each experiment. The test compounds (0.1 ml of 20 mM in ethanol) were added dropwise in the 6 mm glass cylinders fixed in the agar overlay. The antiviral effect was registered on the basis of the difference between the size (diameter in mm) of the zone of plaque inhibition and the zone of cytotoxicity.

Results and Discussion

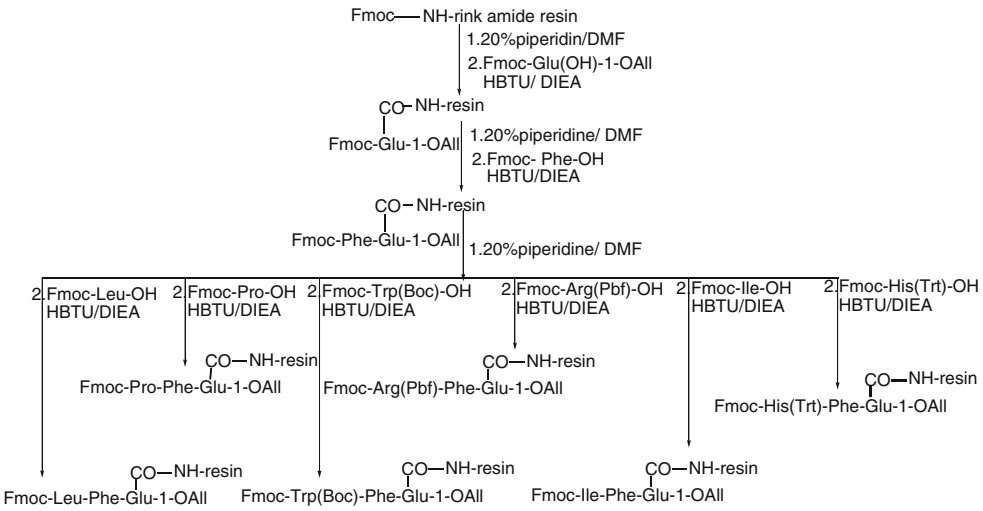
In order to study the possibility to use our established strategy for synthesis of Gln containing peptides and peptides containing pseudopeptide bonds a series of 24 new compounds with a general structure H(or imAc)-Aaa-Phe-Gln-OH(or Im) (Aaa = Arg, Ile, Leu, His, Pro and Trp) were synthesized.

The syntheses of the target peptides were performed by means of solid phase based Multiple-peptide synthesis in parallel.

In all syntheses allyl ester as a protection for α-COOH function of Glu was used. This type of esters was extremely advanced in the last 2–3 years due to their specific properties. First of all, they are quite stable in acidic and alkaline hydrolysis unlike the other ester groups such as OMe, OEt, OBzl and OBu^t, which allows its preservation in all necessary conditions for another protection groups deblocking. Further, this function can be successfully reduced thanks to its similarity with the normal functions of ester to aldehyde group under the action of diisobutylaluminium hydride or to free COOH function using $Pd(PPh)_3/PhSiH_3$. So, this approach allows us to build the peptide chain using the specific features of OAll protection group either to its C- or N-terminus.

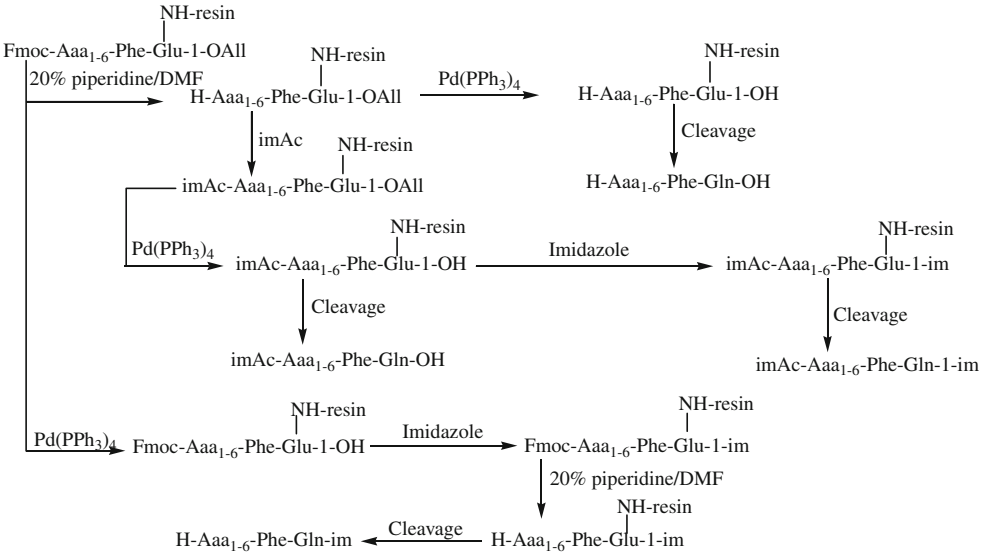
Using Fmoc-Glu(OH)-1-OAll as a first amino acid linked to the resin allowed us to synthesize the general fragment Fmoc-Aaa-Phe-Glu(CONH-resin)-1-OAll that was required for the final products obtaining (Scheme 1).

The main advantage of our strategy is that we use Glu as a first amino acid in place of the Gln residue in the target



Scheme 1 Synthesis of Fmoc-Aaa_{1-6}-Phe-Glu(CONH-resin)-1-OAll

Scheme 2 Synthesis of compounds with general structure: H-Aaa$_{1-6}$-Phe-Gln-im, imAc-Aaa$_{1-6}$-Phe-Gln-OH and imAc-Aaa$_{1-6}$-Phe-Gln-im



Fmoc-Aaa$_{1-6}$-OH: Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-His (Trt)-OH, Fmoc-Trp(Boc)-OH;imA c: 4-Imidazole acetic acid hydrochloride; im: Imidazole;

Table 1 Analytical data for newly synthesized compounds 1–24

No.	Peptide structure	Exact Mass calculated	Mass found	RP-HPLC/Rt (min)
1	H-Arg-Phe-Gln-OH	449.2387	449.98	1.89
2	H-Arg-Phe-Gln-im	499.2656	499.0	1.82
3	imAc-Arg-Phe-Gln-OH	557.2710	558.7	1.72
4	imAc-Arg-Phe-Gln-im	607.2979	608.05	1.74
5	H-Ile-Phe-Gln-OH	406.2216	407.5	1.91
6	H-Ile-Phe-Gln-im	456.2485	457.8	1.76
7	imAc-Ile-Phe-Gln-OH	514.2540	514.6	1.69
8	imAc-Ile-Phe-Gln-im	564.2809	565.5	1.72
9	H-His-Phe-Gln-OH	430.1965	430.4	1.30
10	H-His-Phe-Gln-im	480.2234	480.4	1.09
11	imAc-His-Phe-Gln-OH	538.2288	539.5	2.38
12	imAc-His-Phe-Gln-im	588.2557	588.6	2.60
13	H-Leu-Phe-Gln-OH	406.2216	407.97	1.25
14	imAc-Leu-Phe-Gln-OH	514.2540	514.62	1.17
15	H-Leu-Phe-Gln-im	456.2485	456.5	1.69
16	imAc-Leu-Phe-Gln-im	564.2809	565.48	1.67
17	H-Pro-Phe-Gln-OH	390.1903	390.71	1.32
18	H-Pro-Phe-Gln-im	440.2172	440.1	3.13
19	imAc-Pro-Phe-Gln-OH	498.2227	499.97	1.19
20	imAc-Pro-Phe-Gln-im	548.2496	549.62	1.22
21	H-Trp-Phe-Gln-OH	479.2169	480.77	1.15
22	H-Trp-Phe-Gln-im	529.2438	530.76	1.65
23	imAc-Trp-Phe-Gln-OH	587.2492	589.41	1.96
24	imAc-Trp-Phe-Gln-im	637.2761	639.02	2.04

All MS spectra are included as a supporting information

peptides needed to link the peptide moiety to the rink amide resin. What is more important is that it was linked by its δ-COOH function. The unblocking of Glu using a standard "cocktail" of agents in the final stage of synthesis led to obtaining the required Gln residue (Scheme 2).

Table 2 Antiviral activity screened by the agar-diffusion plaque-inhibition test for compounds **1–18**

No.	Peptide structure	Virus	I Petri	II Petri	Average	Δ (mm)	Effect
6	H-Ile-Phe-Gln-im	LSc-2ab	14 [11]	17 [6.5]	15.5 [8.75]	6.75 mm	±
		CV-B1	0 [10]	0 [8]	0 [9]	–	–
11	imAc-His-Phe-Gln-OH	LSc-2ab	14 [7]	16 [7]	15 [7]	8 mm	±
		CV-B1	0 [6.5]	0 [6.5]	0 [6.5]	–	–
16	imAc-Leu-Phe-Gln-im	LSc-2ab	17 [7]	17.5 [7.5]	17.25 [7.25]	10 mm	±
		CV-B1	0 [7.5]	0 [7]	0 [7.25]	–	–
Ref: OG		LSc-2ab	38 [12]	30 [10]	34 [11]	23 mm	++
		CV-B1	36 [11]	33 [10]	34.5 [10.5]	24 mm	++
Ref: Disoxaril		LSc-2ab	–	–	–	–	–
		CV-B1	25 [11]	25 [11]	25 [11]	14 mm	+
Ref: Guanidine.HCl		LSc-2ab	–	–	–	–	–
		CV-B1	17 [6]	17 [6]	17 [6]	11 mm	+
Ref: HBB		LSc-2ab	25 [6]	25 [6]	25 [6]	19	+
		CV-B1	–	–	–	–	–

(−): $\Delta d \leq 5$ mm; (±): $\Delta d \geq$ 6–10 mm; (+): $\Delta d \geq$ 11–20 mm; (++): $\Delta d \geq$ 21–40 mm

Further the compounds synthesized according to Scheme 1 were used for obtaining new products including imidazole heterocycle. The latter was chosen for the following reasons:

- its incorporation in the desired molecule will allow us to show the possibility to synthesize the peptide moieties including pseudopeptide bonds by means of our strategy,
- it has various biological features including antiviral properties (Katritzky et al. 1984; Grimmett 1997; Brown 1988; Pozharskii et al. 1997; Gilchrist 1985), which could increase the antiviral properties of our mimetics.

4-Imidazole acetic acid hydrochloride (imAc) was used for the incorporation of imidazole moiety into N-terminus of the basic structures. The imidazole (im) was used for the C-terminus modifying (Scheme 2).

The purity of all the newly synthesized compounds was monitored by RP-HPLC and their structure was confirmed by MS spectroscopy (Table 1).

A preliminary antiviral test against *Coxsackieviruses* B1 (CV-B1) and *Poliovirus type 1* (LSc-2ab) using agar-diffusion plaque-inhibition test by means of Oxoglaucine, Disoxaril, Guanidine.HCl and hydroxybenzylbenzimidazole (HBB) as referent compounds (Table 2) were done. For all compounds cytotoxicity was determined in FL cells by using 0.5 l g concentration.

All the rest of the synthesized compounds as well as H-Leu-Phe-Gln-OH and Imidazole have no effect on these two types of viruses according to the agar-diffusion plaque-inhibition test. It was determined that compounds **6**, **11** and **16** are active against CV-B1 and LSc-2ab tested by plaque-inhibition test.

Conclusions

Using multiple peptide synthesis in parallel, a new, original and successful strategy for synthesis of Gln containing peptides using Glu as a starting amino acid was demonstrated and a series of 24 compounds analogues of tripeptide sequence Z-Leu-Phe-Glu-CHO, modified by imidazol moiety were synthesized.

An effective and simple scheme for the inclusion of imidazole heterocycle to C- and/or N-terminus of Gln residue was created by using 1-allyl protecting group as α protecting group for Fmoc-Glu. The approach by means of Fmoc-Glu-1-OAll as a first amino acid linked to the resin could be useful for the synthesis of a large number of compounds consisting of different amids of dicarboxylic amino acids and heterocyclic moieties. Based on the obtained antiviral results we could conclude that some of the newly synthesized compounds showed relatively good antiviral activity against two tested viruses and that the presence of imidazole heterocycle affects the antiviral activity positively according to the tested type of viruses.

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