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Influence of the dipeptide linker configuration on the activity of PSMA ligands

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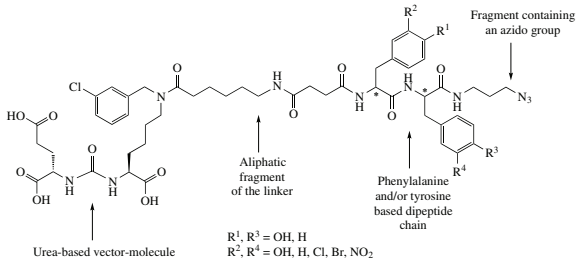
Selective ligands of an urea-based prostate specific membrane antigen with a phenylalanine/tyrosine-based dipeptide linker and with a mingled chiral centers configuration and/or substituted aromatic fragments were prepared in seven steps by liquid- and in six steps by solid-phase synthesis. *In vitro* test for inhibiting the cleavage of *N*-acetylaspartylglutamate revealed the optimum linker containing L-phenylalanine in the structure on the N-terminus of a dipeptide chain.



Keywords: peptide synthesis, target drug delivery, prostate cancer, prostate specific membrane antigen, solid-phase synthesis, amides.

Prostate specific membrane antigen (PSMA) is a promising protein marker for the diagnosis and treatment of prostate cancer.[1] In prostate tumor cells as well as solid vascular tumors, its expression is significantly increased compared to healthy tissues, which makes it promising as a target for drug delivery.[2,3]

Among conjugates for the selective delivery of drugs to prostate cancer cells, the largest number was synthesized with urea-based PSMA inhibitors: DUPA, 2-[3-(1,3-dicarboxypropyl)ureido]pentanedioic acid, and DCL, *N*-{*N*-[(*S*)-1,3-dicarboxypropyl]carbamoyl}-(*S*)-L-lysine.[4] Nowadays, the modification of this structure in order to improve its selective binding to the active center of PSMA is multiply reported.[5,6] One common modification is the introduction of a polypeptide linker into the structure of the ligand, which would interact with the hydrophobic tunnel of PSMA and thus increase its effectiveness.[7,8] The best amino acids for introduction into the peptide chain of the linker were found to be phenylalanine and tyrosine and their substituted analogues.[9,10] However, there are no documented systematized studies and conclusions on the influence of the configuration of chiral centers and the effect of the position of the substituent in the aromatic ring on the biological activity of the linker.[11]

The aim of this work was to synthetize a series of urea-based ligands DCL with an azido group, for which the dependence of the ligand affinity on the position and nature of the substituents in the dipeptide linker was clearly shown. For this purpose, the urea-based fragment of the ligand with the chloro-substituted aromatic fragment at the lysine atom was not varied (Figure 1). The aliphatic fragment of the linker of 6-aminohexanoic acid and succinic anhydride is necessary to ensure the optimal length of the linker. Combination of these two parts is a fixed fragment. This choice was based on previous studies, which showed that such a structure contributes to a high affinity for the active center of PSMA.[12] Phenylalanine- and/or tyrosine-based dipeptide

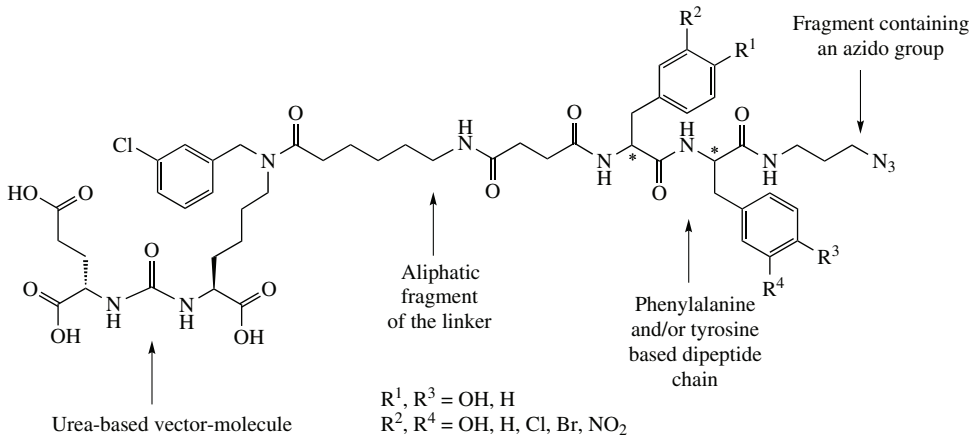


Figure 1 General structure of the ligand.

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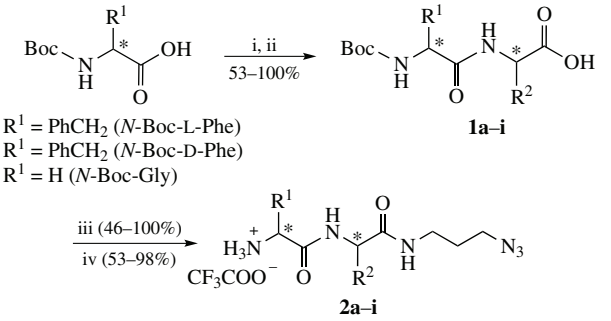


R^1 = $PhCH_2$ (*N*-Boc-L-Phe)
R^1 = $PhCH_2$ (*N*-Boc-D-Phe)
R^1 = H (*N*-Boc-Gly)

a R^1 = $PhCH_2$, R^2 = H (L-Phe-Gly)
b R^1 = H, R^2 = $PhCH_2$ (Gly-L-Phe)
c R^1 = R^2 = $PhCH_2$ (L-Phe-D-Phe)
d R^1 = $PhCH_2$, R^2 = 4-$HOC_6H_4CH_2$ (L-Phe-D-Tyr)
e R^1 = R^2 = $PhCH_2$ (D-Phe-L-Phe)
f R^1 = $PhCH_2$, R^2 = 4-$HOC_6H_4CH_2$ (D-Phe-L-Tyr)
g R^1 = $PhCH_2$, R^2 = 4-$O_2NC_6H_4CH_2$ [L-Phe-L-Phe(4-NO_2)]
h R^1 = $PhCH_2$, R^2 = 3-Br-4-$HOC_6H_3CH_2$ [L-Phe-L-Tyr(3-Br)]
i R^1 = $PhCH_2$, R^2 = 3,4-$(HO)_2C_6H_3CH_2$ [L-Phe-L-Tyr(3-OH)]

Scheme 1 *Reagents and conditions*: i, C_6F_5OH, ECD-Cl, CH_2Cl_2; ii, $H_2NCH(R^2)CO_2H$, DIPEA, THF/H_2O; iii, $H_2N(CH_2)_3N_3$, HBTU, BtOH, DIPEA, THF; iv, TFA, CH_2Cl_2.

chain will vary. The fragment containing an azido group is required for the azide–alkyne addition reaction.

Synthesis of the urea-based vector-molecule fragment was carried out using previously developed methods.[12] The synthesis of the dipeptide chain began with the setting of the Boc-protecting group on the nitrogen atom of the N-terminal amino acid, the removal of this group occurring at the last stage in an acidic solution.[13] We used classical reaction of an amino acid with di-*tert*-butyl dicarbonate in an alkaline solution. At the second step, we synthesized dipeptides **1a–i** (Scheme 1, stages i and ii).[14] An activated N-terminal amino acid pentafluorophenyl ester was initially prepared and subsequently reacted with the free amino acid.[15] The resulting compounds were sufficiently pure to use for further synthesis steps. In the third step, the activation the carboxyl group of dipeptides with HOBt and HBTU activating agents was conducted with the following reaction with 3-azidopropylamine (see Scheme 1, stage iii). The substances were purified by extraction followed by column chromatography. The *tert*-butoxycarbonyl protecting group was removed to obtain compounds **2a–i** using a 10% solution of trifluoroacetic acid in dichloromethane (stage iv). In this manner, nine dipeptide linkers were synthesized, which were further used to produce highly specific PSMA vectors. These methods provide high yields and easy purification of the products.

The synthesis of ligands with dipeptide linkers included well-recognized peptide coupling reaction. Catalytic reagents HOBt and HBTU led to formation of activated esters, which reacted readily with amino acids.[16] (Scheme 2, stage i).

For binding with PSMA, the ligands require the presence of free carboxyl groups.[11] To remove *tert*-butyl protecting groups, conversion of *tert*-butyl esters to carboxylic acids was carried out in a system: 46.25% TFA, 46.25% DCM, 5% water and 2.5% TIPS (see Scheme 2, stage ii). Triisopropylsilane acts as a cationic scavenger and protects the side chains of peptide from undesired parallel reactions.[17,18] Amino acid sequences are sensitive to strong acid conditions, so the concentration of trifluoroacetic acid was reduced to 45–50%.[19] Hence, a number of PSMA inhibitor ligands **3a–i** was prepared. The obtained compounds were characterized by NMR spectroscopy, high-resolution mass spectrometry and high-performance liquid chromatography.

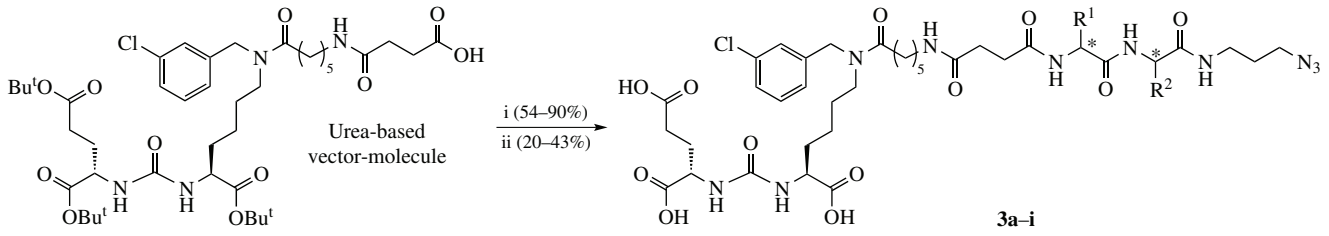
When attempting an introduction of the amino acid tyrosine from the N-terminus of the dipeptide sequence by liquid phase synthesis methods, a number of difficulties were found associated with poor solubility of tyrosine, as well as side reactions from the hydroxyl group in the *para* position. We tried a different approach to produce the desired dipeptide chains, namely, *viz*., a solid-phase synthesis (*cf*. ref. 20).

In this work, 2-chlorotritile resin in chloride form (2-CTC) was selected as the solid support. This resin prevents the racemization of the first amino acid residues and provides the possibility of using a wide variety of side chains. It is also important to note the conditions for removing the resulting chains from the resin.[21,22] Weakly acidic conditions allow for maintaining *tert*-butyl protecting groups present in the structure of both the molecule vector and the linker.

Fmoc-protected amino acid residues of phenylalanine and tyrosine were used for synthesis. Removal of Fmoc protection takes place under basic conditions, which corresponds to the type of resin chosen. At the first stage, we activated the resin and introduced the first amino acid residue (tyrosine). Chain building was performed by creating a peptide bond between the amino group of the residue on the resin and the carboxyl group of the following Fmoc-protected amino acid residue.

A modified urea-based vector-molecule was attached to the obtained dipeptide chain after removal of Fmoc protection group by creating a peptide bond using activated HOBt and HBTU esters. Removal from the resin was carried out in a 0.5% solution of trifluoroacetic acid in dichloromethane. The *tert*-butyl protecting groups of the vector-molecule and the tyrosine fragment in the linker side chain were not affected.

Since the azide–alkyne cycloaddition reaction requires the presence of an azide group in the ligand structure, the next step involved the reaction of compound **4** with 3-aminopropyl azide (Scheme 3, stage i). To avoid racemization and minimize the amount of by-products, it is important to consider the addition order of the reagents. The reaction was executed without preactivation and at a reduced temperature (0 °C). The removal of *tert*-butyl protecting groups was carried out in the standard way (stage ii). The obtained compound **5** was characterized by ^{1}H NMR, HPLC-MS and HRMS spectra.



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b R^1 = H, R^2 = $PhCH_2$ (Gly-L-Phe)
c R^1 = R^2 = $PhCH_2$ (L-Phe-D-Phe)
d R^1 = $PhCH_2$, R^2 = 4-$HOC_6H_4CH_2$ (L-Phe-D-Tyr)
e R^1 = R^2 = $PhCH_2$ (D-Phe-L-Phe)
f R^1 = $PhCH_2$, R^2 = 4-$HOC_6H_4CH_2$ (D-Phe-L-Tyr)
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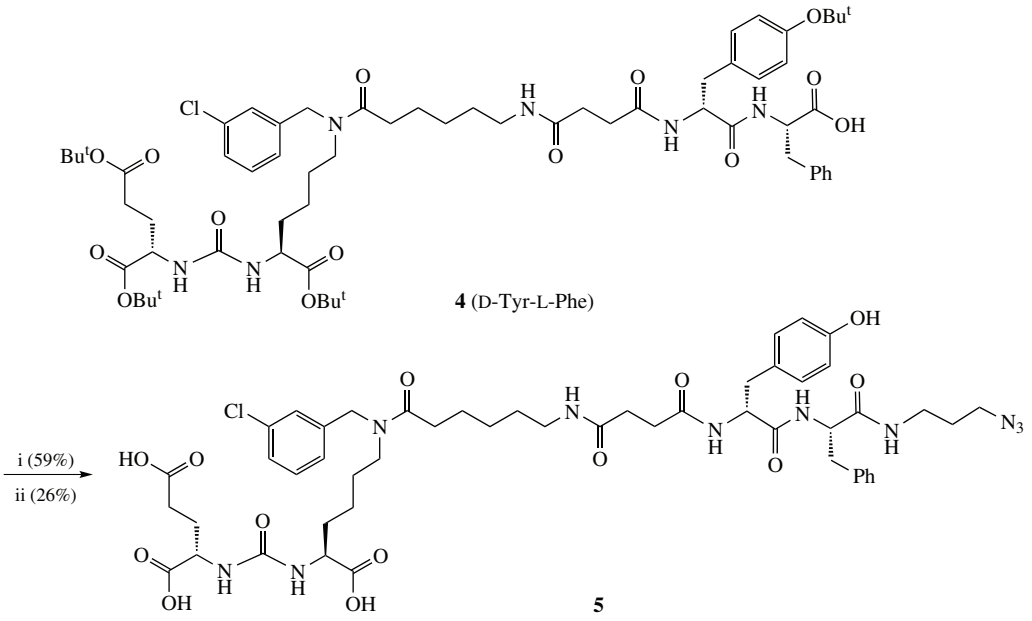
h R^1 = $PhCH_2$, R^2 = 3-Br-4-$HOC_6H_3CH_2$ [L-Phe-L-Tyr(3-Br)]
i R^1 = $PhCH_2$, R^2 = 3,4-$(HO)_2C_6H_3CH_2$ [L-Phe-L-Tyr(3-OH)]

Scheme 2 *Reagents and conditions*: i, dipeptide **2a–i**, HOBt/HBTU, DIPEA, DMF; ii, TFA–CH_2Cl_2 (1 : 1), H_2O (5%), TIPS (2.5%).



Scheme 3 *Reagents and conditions*: i, $H_2N(CH_2)_3N_3$, HOBt, HBTU, DMF, 0 °C; ii, TFA–CH_2Cl_2 (1 : 1), H_2O (5%), TIPS (2.5%).

The affinity of the synthesized vectors with the linker correlates with the efficiency of inhibiting their cleavage of PSMA *N*-acetylaspartylglutamate. After analyzing several cell lines available in our laboratory and according to available data, it was found that the highest level of PSMA was observed in the LNCaP cell line, which was taken for further work.[23,24] Control test was conducted with the DCL drug analogue synthesized in our laboratory. Table 1 shows the results of a series of 10 new ligands by inhibiting the cleavage reaction of *N*-acetylaspartylglutamate on the cell line LNCaP as well as data on ligands obtained earlier.[12] In the structure of the latter, the molecule vector is also represented by urea DCL with a *m*-chloro-substituted aromatic fragment at a lysine atom.

According to the obtained data, the presence of an aromatic fragment in the C-terminus of the linker is critical and strongly affects the affinity of the ligand L-Phe-Gly (821.0 ± 162.0 nM). The absence of an aromatic fragment in the N-terminal position also negatively affects affinity, but not so pronounced (Gly-L-Phe, 293.0 ± 37.2 nM). It can be concluded that the number and position of aromatic fragments in the linker structure has a significant role in biological activity, so the presence of dipeptide aromatic fragments is required to maintain a high affinity for PSMA.

Table 1 Results of IC_{50} affinity parameter determination by inhibiting the cleavage reaction of *N*-acetylaspartylglutamate.

Linker structure	IC_{50} ± SD[a]/nM[b]	K_i ± SD[a]/nM[b]
	Control	
DCL	2149.0 ± 235.0	224.6 ± 24.6
	Ligand Series Gly/Phe	
Gly-L-Phe (**3b**)	293.0 ± 37.2	30.2 ± 3.83
L-Phe-Gly (**3a**)	821.0 ± 162.0	84.6 ± 16.7
	Ligand Series Phe-Tyr(Phe)	
L-Phe-L-Tyr	22.5 ± 6.0	2.4 ± 0.6
D-Phe-D-Phe	58.0 ± 16.3	6.06 ± 1.7
L-Phe-L-Phe	86.2 ± 23.9	9.01 ± 2.5
L-Phe-D-Phe (**3c**)	279.0 ± 37.3	28.8 ± 3.84
D-Phe-L-Phe (**3e**)	520.0 ± 111.0	53.7 ± 11.5
D-Phe-D-Tyr	820.0 ± 447.0	85.7 ± 46.7
L-Phe-D-Tyr (**3d**)	1080.0 ± 179.0	112.0 ± 18.5
D-Phe-L-Tyr (**3f**)	1330.0 ± 307.0	137.0 ± 31.6
	Ligand Series Tyr-Tyr(Phe)	
D-Tyr-L-Phe (**5**)	741.0 ± 123.0	76.5 ± 12.7
	Ligand Series with substituted Phe/Tyr	
D-Phe-D-Tyr(Br)	267.3 ± 151.8	27.9 ± 15.9
L-Phe-L-Phe(NO_2) (**3g**)	959.0 ± 107.0	100.3 ± 11.2
L-Phe-L-Tyr(Br) (**3h**)	1390.0 ± 341.0	143.0 ± 35.2
L-Phe-L-Tyr(3-OH) (**3i**)	6030.0 ± 1140.0	622.0 ± 117.0

[a] SD – standard deviation. [b] nM – nanomolar.

Ligands with an unmixed structure show the best results, in particular, the dipeptide chain L-Phe-L-Tyr reveals the highest affinity (22.5 ± 6.0 nM). Dipeptide chains with the mixed structure are inferior in affinity, but it is possible to conclude that L-phenylalanine has a favorable effect on affinity. The highest values belong to ligands containing L-phenylalanine from the N-terminus of the chain: L-Phe-L-Tyr (22.5 ± 6.0 nM), L-Phe-L-Phe (86.2 ± 23.9 nM), L-Phe-D-Phe (279.0 ± 37.3 nM). For ligand D-Tyr-L-Phe, the IC_{50} value of 741.0 ± 123.0 nM indicates that the introduction of tyrosine into the position 1 of the dipeptide chain reduces affinity.

The introduction of the second hydroxy group at the position 3 of the tyrosine moiety turned out to be negative for affinity and practically makes the molecule inactive. However, the results for D-Phe-D-Tyr(3-Br) (267.3 ± 151.8 nM) and L-Phe-L-Tyr(3-Br) (1390.0 ± 341.0 nM) show that the configuration has a strong effect on affinity.

In conclusion, a series of ten novel PSMA ligands bearing dipeptide linkers with mixed chiral site configurations and/or substituted aromatic moieties were obtained. A new solid-phase synthesis of such ligands proved to be very efficient. Based on the results of *in vitro* biological tests, it can be concluded that ligands with a mixed-configured dipeptide linker are inferior to ligands with non-mixed ones, the best result for the new series relating to L-Phe-D-Phe (279.0 ± 37.3 nM) dipeptide chain. Substituents in aromatic fragments in the linker structure can also have a significant effect on affinity, but it is inextricably linked to the configuration of the amino acid residue, which is promising for further study.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2020.11.022.

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