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Mendeleev Commun., 2023, **33**, 472–475

Mendeleev Communications

Choice of the optimal synthetic approach for the polypeptide ligands of prostatic specific membrane antigen preparation

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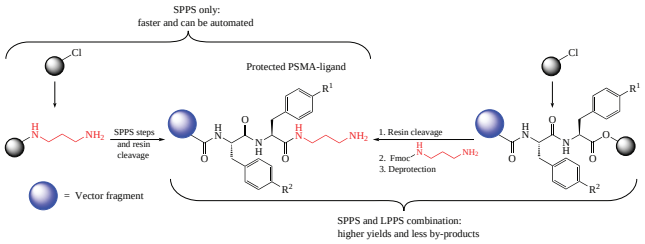
DOI: 10.1016/j.mencom.2023.06.010

Two alternative synthetic schemes involving solid-phase peptide synthesis steps for the preparation of prostatic specific membrane antigen ligands based on Glu-Urea-Lys with peptide fragments in the linker structure are compared. In the first approach, the amino acid key intermediate is attached to the 2-CTC resin by the carboxy group while in the second approach, by the amino one. The preference for each approach is dependent on the particular target molecule.



Keywords: solid-phase peptide synthesis, prostatic specific membrane antigen, prostate cancer, targeted delivery, anticancer drugs.

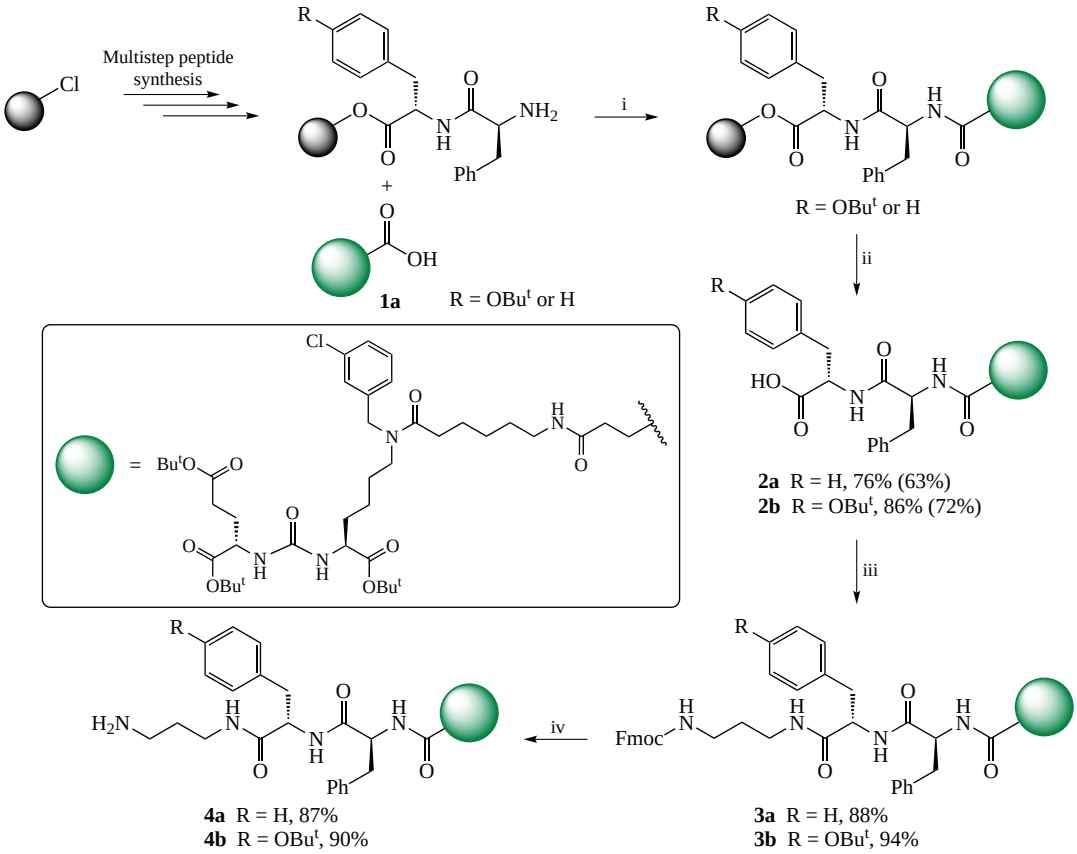
Prostate cancer (PCa) is the second most common cancer among men.[1] In recent years, an approach to therapy and diagnosis of prostate cancer based on targeted drug delivery to diseased cells through selective binding to prostate-specific membrane antigen (PSMA), which is hyperexpressed in PCa cells has been actively developed.[2–9] However, due to the structural complexity of the molecules to be synthesized, existing methods for their syntheses are labor-intensive and consist of multi-step procedures using both liquid- and solid-phase peptide syntheses and chromatographic separation of the products in most steps.[6,10] Thus, the choice of optimal highly efficient and less time-consuming synthetic strategy for the preparation of PSMA-targeted ligands is an urgent task.

The aim of this work was to comparatively analyze two synthetic routes (Schemes 1 and 2) to obtain PSMA ligands **4a–i** and **8** suitable for subsequent conjugation with different therapeutic or diagnostic agents, which can be chelating agents,[11,12] fluorescent labels[13,14] and other functional fragments. Various options for conjugating ligands and different biologically active agents have been presented in the literature,[15,16] and the most common method is to link the PSMA-vector and functional fragments through an amide bond; therefore, the synthesis of PSMA ligands with terminal carboxy or amino groups for subsequent amide production seems rational. A comparative analysis of the two alternative approaches was carried out and, based on this analysis, the limits of applicability of each strategy for different target compounds were determined.

In one of the approaches, we used the pre-functionalization of 2-chlorotrityl chloride (2-CTC) resin with 1,3-diaminopropane. When using solid-phase synthesis techniques to produce PSMA ligands, the amino acid fragment is most commonly immobilized on 2-CTC resin with the C-terminal fragment,[6,17,18] but in this work we used the N-terminal fragment as the first link in the polypeptide chain. In the previous works,[19–21] the authors immobilized the 1,3-diaminopropane fragment using preoperatively simple methods, but after synthesizing the peptide sequence with the 1,3-diaminopropane fragment on the resin, its removal was carried out under harsh conditions in 95% trifluoroacetic acid (TFA). Such reaction conditions lead to the removal of all acid-labile protecting groups from the molecule, so this approach is unacceptable for the compounds in cases where acid-labile groups must be partially preserved, *e.g.*, for the synthesis of target protected PSMA ligand **4b** (see Scheme 1). Thus, while this approach is promising, it required significant optimization in order to retain the necessary protective groups.

The synthesis of vector fragments **1a–d** (see Scheme 1) was carried out using the previously described techniques.[5,22] These vector fragments were selected based on the fact that a wide range of PSMA ligands as well as conjugates with therapeutic and diagnostic agents have previously been derived from them.[5,8] Dipeptides with two L-phenylalanine residues as well as L-phenylalanine and L-tyrosine residues were chosen as model peptide linkers. The choice of the L-isomers of amino acid residues was made based on the data showing that the best affinity for ligands of similar structure is achieved in the case of L configuration.[23] The hydroxy group of tyrosine was protected by a *tert*-butyl group.

The target ligands **4a**,**b** were synthesized by two alternative methods (see Schemes 1 and 2). The key difference between the synthetic schemes was that in Scheme 1, the starting reagent was attached to the resin *via* the carboxy fragment, and the subsequent transformations were the combination of solid-phase peptide synthesis and reactions in solution. In Scheme 2, the starting reagent was attached to the resin *via* the amino group, so the



Scheme 1 *Reagents and conditions*: i, HBTU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate), HOBt (1-hydroxybenzotriazole), DIPEA (*N*,*N*-diisopropylethylamine), DMF; ii, 0.75% TFA/CH_2Cl_2 (v/v); iii, FmocNH$(CH_2)_3NH_2$, HBTU, HOBt, DIPEA, DMF; iv, Et_2NH, DMF.

ligand assembly was entirely performed on the resin. According to the first methodology, the first amino acid fragment was attached to the CTC-2 resin to form an ester bond, and then the dipeptide sequence was assembled in a solid-phase manner using the Fmoc-strategy.[6,17,18] The dipeptide was acylated with vector fragment **1a** on the resin, and then the resulting compounds **2a**,**b** were removed from the solid carrier. Further acylation of mono-Fmoc-1,3-diaminopropane with products **2a**,**b** gave compounds **3a**,**b**, and the removal of the Fmoc-protective groups from them led to the target ligands **4a**,**b**.

The overall yield of the proposed Scheme 1 in the case of compound **4a** is 58% relative to the resin capacity and 48% relative to the vector fragment **1a**, and in the case of compound **4b** it is 73% relative to the resin capacity and 61% relative to compound **1a**. In stages ii (removal from the resin), iii (acylation of FmocNH$(CH_2)_3NH_2$) and iv (removal of Fmoc group), the isolation of the products **2**–**4** was performed using column chromatography.

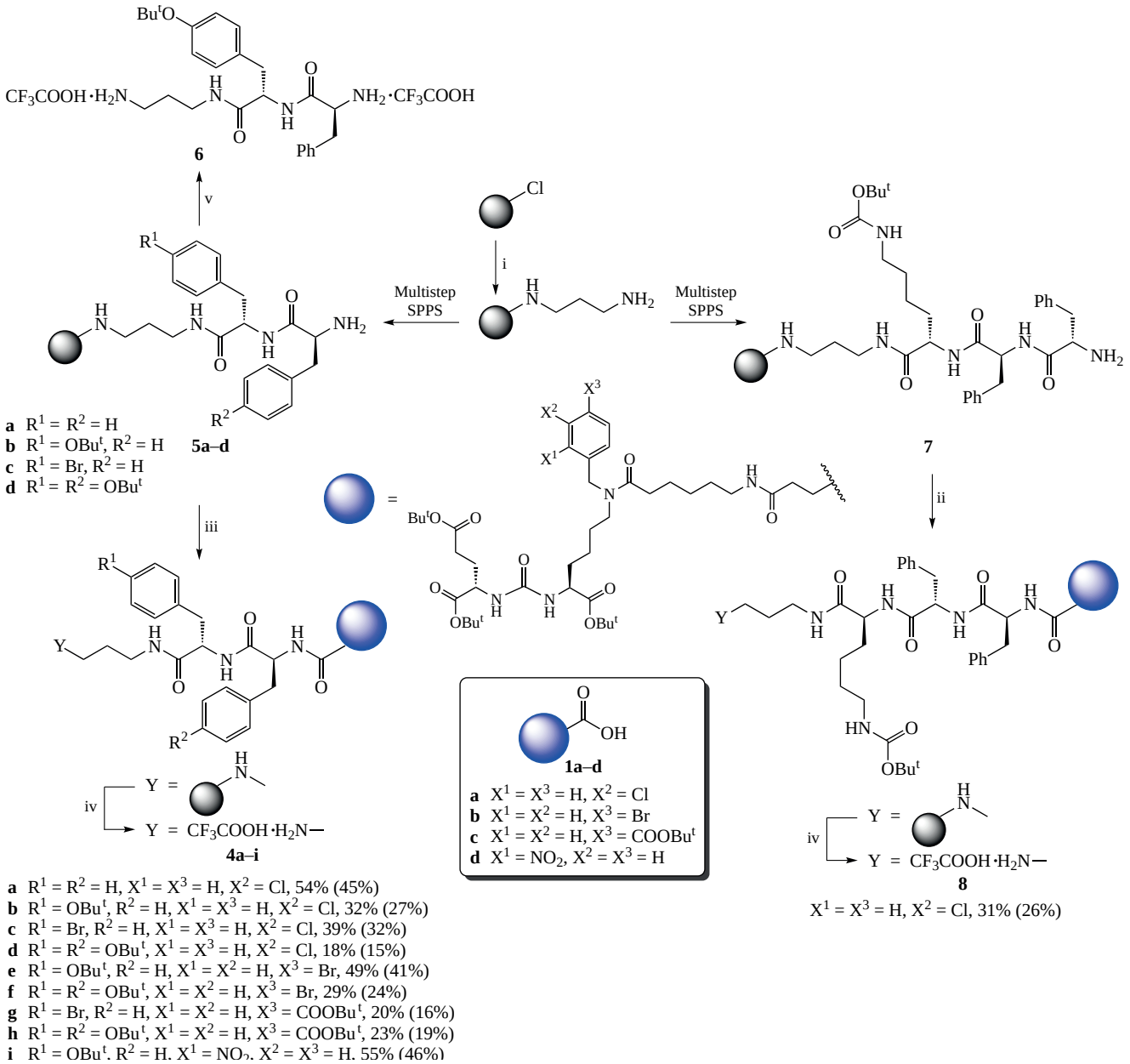
The second synthetic Scheme 2 involved initial immobilization of 1,3-diaminopropane on STS-2 resin, followed by solid-phase peptide synthesis (SPPS).[21] Modified peptide fragments **5a**–**d** were produced in this way. When using solid-phase synthesis techniques to produce PSMA ligands, the amino acid fragment is usually immobilized on 2-CTC resin with the C-terminal fragment.[6,17,18] In this synthetic route, however, the N-terminal fragment was used as the first link in the polypeptide chain.

To confirm that the immobilized fragment can be effectively removed from the resin, a test reaction was carried out with compound **5b**. In earlier reports,[19–21] the authors immobilized the 1,3-diaminopropane fragment using simple methods but removed the protecting groups under harsh conditions with 95% TFA. Such processing caused removal of all acid-labile protecting groups, so it is not applicable in cases where it is necessary to preserve them in the final product. Thus, to apply this synthetic approach to the target PSMA ligands **4a**–**i**, an optimization providing preservation of the *tert*-butyl groups in the molecules was required. This was achieved by removing products **4a**–**i** from the resin with a 0.75% TFA solution in CH_2Cl_2. It should be noted that removal from the resin in this case was slower than in the case when the peptide was immobilized with the C-terminal fragment. Subsequently, a model reaction for the preparation of protected ligand **4e** was carried out using the vector fragment **1b** and immobilized modified peptide **5b**. The target compound **4e** was isolated by column chromatography in 51% yield relative to the resin and 36% yield relative to the vector molecule **1b**.

To estimate the synthetic prospects of Scheme 2, a series of ligands **4a**–**c**,**d**,**f**–**i** were synthesized based on vector molecules **1a**–**d**. Nevertheless, high yields comparable to the total yields of the synthetic Scheme 1 were achieved only in the case of ligands **4a** (yield relative to the vector fragment according to Scheme 2 is 43% *vs*. 48% according to Scheme 2), **4e** and **4i**. High yields could not be reached for compounds **4c**,**d**,**f**,**g** containing peptide fragments with phenylalanine and bromo-substituted phenylalanine residues or two tyrosine residues.

We also evaluated the applicability of the proposed strategy to the synthesis of protected ligand **8**, suitable for the synthesis bimodal conjugates (see Scheme 2).[6] Compound **8** was obtained in only about 21% yield; according to HPLC-MS, it appears to be contaminated with a product containing no Boc-protective group. It may be concluded that the proposed method is not a suitable alternative for the synthesis of PSMA ligands of similar structure.

In general, the first synthetic route (see Scheme 1) is more labor-intensive due to two additional steps with chromatographic separation of the products (steps for compounds **3a**,**b** and



a $R^1 = R^2 = H$, $X^1 = X^3 = H$, $X^2 = Cl$, 54% (45%)
b $R^1 = OBu^t$, $R^2 = H$, $X^1 = X^3 = H$, $X^2 = Cl$, 32% (27%)
c $R^1 = Br$, $R^2 = H$, $X^1 = X^3 = H$, $X^2 = Cl$, 39% (32%)
d $R^1 = R^2 = OBu^t$, $X^1 = X^3 = H$, $X^2 = Cl$, 18% (15%)
e $R^1 = OBu^t$, $R^2 = H$, $X^1 = X^2 = H$, $X^3 = Br$, 49% (41%)
f $R^1 = R^2 = OBu^t$, $X^1 = X^2 = H$, $X^3 = Br$, 29% (24%)
g $R^1 = Br$, $R^2 = H$, $X^1 = X^2 = H$, $X^3 = COOBu^t$, 20% (16%)
h $R^1 = R^2 = OBu^t$, $X^1 = X^2 = H$, $X^3 = COOBu^t$, 23% (19%)
i $R^1 = OBu^t$, $R^2 = H$, $X^1 = NO_2$, $X^2 = X^3 = H$, 55% (46%)

Scheme 2 *Reagents and conditions*: i, $H_2N(CH_2)_3NH_2$, CH_2Cl_2; ii, **1a**, HBTU, HOBt, DIPEA, DMF; iii, **1a–d**, HBTU, HOBt, DIPEA, DMF; iv, 0.75% TFA/CH_2Cl_2 (v/v); v, 0.75–1% TFA/CH_2Cl_2 (v/v).

4a,**b**). However, this method gives higher total yields of the target products: 48% for compound **4a** and 61% for **4b** (yield values calculated relative to vector molecule **1a**). The alternative synthetic Scheme 2 is less labor-intensive, since chromatographic separation is only applied after the target compounds have been removed from the solid-phase carrier, and has the potential to be automated using a peptide synthesizer. However, the yield of the target products is generally lower than that in the first synthetic scheme, although in some cases (namely, in the absence of acidic labile functional groups in the peptide fragment) comparable yields can be achieved. Also, the solid-phase method shown in Scheme 2 is more difficult to scale up. A comparison of the proposed methods is summarized in Table 1.

In conclusion, when obtaining precursor compounds of PSMA ligands suitable for the synthesis of conjugates through the amide bonds formation, the optimum is: 1) a combination of solid-phase and liquid-phase peptide synthesis with initial attachment of the reagent to the resin at the carboxy group, if the aim is to obtain significant quantities of the target compound in maximum yield, or to synthesize compounds suitable for bimodal conjugates; 2) the use of solid-phase peptide synthesis with initial attachment of the reagent to the resin at the amino group, if the aim is to quickly build up a library of compounds. Also, in this work a method has been proposed for the synthesis on 2-CTC resin with an immobilized amino fragment of compounds protected by acid-labile groups, suitable for subsequent functionalization after removal from the solid phase carrier.

Table 1 Comparison of efficiency for synthetic Schemes 1 and 2.

Product	Scheme 1				Scheme 2			
	Number of steps			Yield[c] (%)	Number of steps			Yield[c] (%)
	Total	With SPPS[a]	With CS[b]		Total	With SPPS[a]	With CS[b]	
4a	8	5	3	58/48	7	6	1	54/5
4b	8	5	3	73/61	7	6	1	32/27

[a] Solid-phase peptide synthesis. [b] Chromatography separation. [c] Based on the resin / vector fragment **1a**.

The research was supported by Russian Science Foundation (grants nos. 22-15-00098, https://rscf.ru/project/22-15-00098/,

Development of a method to produce PSMA ligands using a solid-phase technique, and 22-73-00066, https://rscf.ru/project/22-73-00066/, Method validation for creating ligands with bimodal functionalization capability).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.06.010.

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Received: 27th March 2023; Com. 23/7132