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Letter

Enzymatic acylation of nucleosides—Novel route to nucleopeptides

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ABSTRACT

An efficient and straightforward proteolytic enzyme-catalyzed approach towards the regioselective synthesis of nucleopeptides was developed. Appropriate reaction conditions were investigated and certain peptide-modified nucleosides were obtained with 70–90% yields. The obtained compounds could be efficiently used for medicinal diagnostic kits and therapeutic treatment.

1. Introduction

Nucleopeptides and peptide-oligonucleotide conjugates have been of great interest to researchers over the last decade due to their unique combination of outstanding properties. Relevant application in many fields of high technology can be found for nucleotides and oligonucleotides containing peptidyl moieties: organic synthesis of natural products, pharmaceuticals, drugs, etc. They are used for native DNA/RNA labeling in order to establish the dimensional structure and hybridization properties of nucleic acids [1,2] and as markers for synthetic polynucleotides [3]. Synthetic oligonucleotides bearing arginine-containing peptidyl modified centers are reported to be immune-stimulating agents and anti-cancer reagents [4]. Shvachkin and coworkers briefly reported anti-nociceptive activity of synthetic nucleopeptides [5]. Recently the applicability of a peptide-nucleotide dual vaccine for inhibition of human telomerase reverse transcriptase (hTERT) was described; therefore nucleopeptides bear out significant promise as anti-cancer therapeutic agents [6]. It was also demonstrated that nucleopeptides could be efficiently used for enhanced targeted delivery of therapeutic agents, antisense and siRNA into living cells for medical treatments [7–9].

Therefore, we set out to devise a straightforward and efficient synthetic approach in the hope that it could be amenable for the preparation of novel nucleopeptides.

2. Materials and methods

2.1. Enzyme and chemicals

Subtilisin-72 was isolated and purified by common methods from *Bacillus subtilis* strain 72. All other chemicals used were of analytical grade and purchased from Sigma–Aldrich and Serva.

2.2. Enzymatic reactions

2.2.1. Representative experimental procedure for the synthesis of 3′-O-(Dnp-AlaAlaLeu)-dT

A mixture of Subtilisin-72 (10 μg) and 2′-deoxythymidine **1a** (10 μmol) was dissolved in 500 μl of phosphate buffer (pH 7.4). The resulting solution was frozen and lyophilized. The lyophilisate obtained was dissolved in 120 μl of phosphate buffer (pH 7.4) and digested dropwise on the surface of Silochrom-C-80 in a reaction vial. The sorbent was dried in high vacuum over phosphorus pentoxide. Next, Dnp-AlaAlaLeu-OMe **2** (20 μmol) was dissolved in THF (1 ml), pyridine (50 μl) was added and the resulting solution was added to a reaction vial with dried sorbent, the vial was capped and the whole reaction mixture was stirred at room temperature (approx. 18–23 °C) for 96 h. After the reaction was complete, the solvent was separated from the solid phase and evaporated. Acetonitrile (3 ml) was added to the residue, undissolved precipitate was discarded. The solution obtained was purified with RP-HPLC (30–60% acetonitrile in water + 0.1% TFA gradient) and the target compound 3′-O-(Dnp-AlaAlaLeu)-dT **3a** was obtained in 89% yield as a fine yellow powder. SALDI-MS: 664.3 $[M+H]^+$; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 1.1(s), 1.4–1.6 (dd), 1.8–2.2(m), 2.4–2.5(m), 3.5–3.8(m), 4.3–4.4(m), 4.9(m), 5.8(t), 6.7–6.8(dd), 7.6(s), 8.0(2d), 8.3–8.9 (d + s), 10.1(s) ppm.

2.3. Analytical methods

The aminolysis reaction was monitored by RP-HPLC analysis carried out in a system (Beckman System Gold) composed of a column (Beckman C18 5 μM, 250 mm × 4.6 mm) and a UV/vis detector (Beckman System Gold, modul167, λ = 220 nm). The compounds were eluted by gradient elution with an eluent system of 5% acetonitrile/H_2O (A) and 95% acetonitrile/H_2O (B), both containing 0.05% of trifluoroacetic acid (see Table 1).

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Table 1
Gradient elution for HPLC analysis.

Time, min	Flow rate, ml/min	% A	% B
0	1	80	20
10	1	40	60
15	1	40	60
20	1	80	20

Table 2
Starting materials and yields of novel nucleopeptides **3a**–**d**.

#	Nucleoside	Nucleopeptide	Yield, %
3a	dT	3′-O-(Dnp-AlaAlaLeu)-dT	89
3b	dC	3′-O-(Dnp-AlaAlaLeu)-dC	87
3c	dA	3′-O-(Dnp-AlaAlaLeu)-dA	71
3d	dG	3′-O-(Dnp-AlaAlaLeu)-dG	77

3. Results and discussion

3.1. The reaction parameters

In order to run enzymatic reactions in non-conventional media (organic solvent) it is necessary to prevent the enzyme inactivation. We used the enzyme that had been adsorbed on the surface of Silochrom-C-80. Adsorbtion, followed by lyophilization, leads to formation of a monolayer of enzyme with incorporated water molecules. Thus, incorporated water supplies enough humidity for enzyme to stay active in organic solvent.

3.2. Choice of enzyme

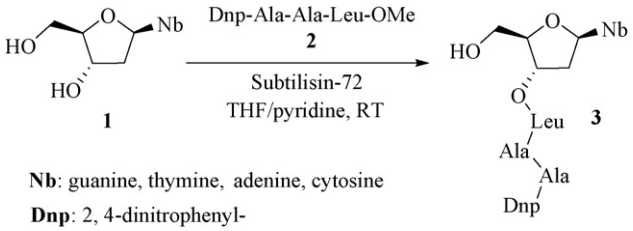
Subtilisin-72, which is closely related to the well characterized subtilisin Carlsberg, has a rather broad specificity. A number of reactions for peptide bond synthesis catalyzed by Subtilisin-72 were described. Moreover, we have shown that the enzyme's S'_1-subsite is capable of accepting different nucleophilic components, not only N-nucleophiles (amines, amino acids and peptides) but also O-nucleophiles (alcohols, sugars).

3.3. Known synthetic procedures

Different approaches to the nucleopeptide synthesis have been suggested, but generally these methods are too complicated. The most used synthetic pathways to the nucleopeptides are those based on solid-phase synthesis [10], and pure chemical preparations, exemplified by different authors, for example EDCI/HOBt coupling [11–13]. Basically, all the synthetic procedures described so far are based on multi-step synthesis involving employment of protective groups. Also enzymatic catalysis is employed, using lipases from different species (*i.e. Candida antarctica*, *Pseudomonas sepacia*). These lipases show pretty good results, but lipase-catalysis is widely investigated and is broadly used. The novelty of our work is that we prove the use of the proteolytic enzyme Subtilisin-72 with an uncommon acyl-acceptor of non-amino acid nature.

3.4. Results for peptidoacylation of nucleosides

Recently we reported the efficient enzyme-catalyzed synthesis of various peptidyl-amides using Subtilisin-72/Silochrom as a catalyst [14]. Here we desired to develop a more efficient synthetic route by using the solid-phase-supported enzyme in non-conventional medium to catalyze the reaction of direct acylation of unprotected nucleosides. We employed the combined methods of Wong and Fang [15] and Rich and Dordick [16], and have modified and improved these methods to achieve the desired results. It was found that experiments with unchanged methods (*i.e.* without lyophilization and sorption) do not lead to the desired target molecule but only a peptide hydrolysis product. It was found that the acylation reaction between non-activated esters (peptidyl methyl ester) and free nucleosides (**3a**–**d**, Table 2) proceeded smoothly at room temperature in high yields when an acylating agent was lyophilized together with the enzyme and deposited on the surface of Silochrom-C-80 to prepare the catalyst. The general procedure is shown in Scheme 1.



Scheme 1.

In the course of our investigations we have tried different solvents, namely, acetonitrile, THF and 1,4-dioxane. It was shown that reactions in acetonitrile and THF produced the target compounds in almost the same yields, while no reaction occurred if dioxane was used as a solvent. Also pyridine was added to the reaction medium for better solubility of starting nucleoside.

It was shown that coupling of **1** with peptide **2** proceeded regioselectively. The formation of 3′-O-acylation products was observed while no N-substitution of peptido-nucleosides took place. Reaction products are shown in Table 2, NMR-spectra and appearance are shown in Table 3.

Surprisingly, further experiments demonstrated that under the described conditions only acylation of deoxyribonucleosides occurred. All attempts to modify ribonucleosides, ribo- or deoxyribonucleotides with the peptide failed. We believe that in the case of these compounds no coupling between target hydroxyl and the enzyme's active center took place due to sterically hindered phosphate groups of nucleotides and ribo-hydroxyl groups. The obtained novel compounds are shown in Tables 2 and 3 with appearance, reaction yields and spectroscopic data.

Table 3
Physical data of novel nucleopeptides **3a**–**d**.

#	Color	^{1}H NMR[a] δ ppm	MS[b]
3a	Yellow solid	1.1(s), 1.4–1.6(dd), 1.8–2.2(m), 2.4–2.5(m), 3.5–3.8(m), 4.0(s), 4.3–4.4(m), 4.9(m), 5.8(t), 6.7–6.8(t), 7.6(s), 8.0(2d), 8.3–8.9(d), 10.1(s)	664.3
3b	Yellow solid	1.1(s), 1.3–1.6(dd), 1.8–2.1(m), 2.4–2.6(m), 3.4–3.7(m), 4.0(s), 4.3–4.4(m), 5.8(t), 6.7–6.9(t), 7.3(s), 8.0(dd), 8.3(d), 8.9(s)	649.7
3c	Yellow solid	1.2(s), 1.4–1.6(dd), 1.7–1.9(m), 2.1(s), 2.3–2.6(m), 3.5–3.7(m), 4.0(s), 4.3–4.5(m), 5.8(t), 6.6–6.9(t), 8.0(dd), 8.1(s), 8.3(d), 8.7(s), 8.9(s)	673.3
3d	Yellow solid	1.0(s), 1.3–1.5(dd), 1.7–1.8(m), 2.1(s), 2.5–2.8(m), 3.4–3.8(m), 4.0(s), 4.2–4.4(m), 5.9(t), 6.7–6.9(t), 7.9–8.1(dd), 8.4(d), 8.9(s)	689.6

[a] ^{1}H NMR was registered in DMSO-d_6 at 300 MHz.
[b] SALDI-MS: $[M+H]^+$.

4. Conclusions

A novel and highly efficient method was developed for the synthesis of peptide-modified deoxynucleosides under mild conditions under proteolytic enzyme catalysis. Regioselectivity of the reaction was investigated; formation of 3′-O-acylation products was established. Biological evaluation of the synthesized peptides is ongoing.

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