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TOTAL SYNTHESIS OF THE ACTIVE INGREDIENT ACETYL TETRAPEPTIDE-3 FOR HAIR LOSS TREATMENT AND SCALP CARE

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Abstract. Acetyl tetrapeptide-3, a raw material for cosmetic production, was successfully synthesized on the laboratory scale with a high level of purity using the solid-phase peptide synthesis technique. Analysis by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) confirmed that the yield of acetyl tetrapeptide-3 was over 56% with a purity of 100%. This study supports the development of hair loss and scalp care products incorporating acetyl tetrapeptide-3 as a key active agent.

Keywords: acetyl tetrapeptide-3, cosmetic peptides, solid phase peptide synthesis, anti-hair loss.

1. Introduction

Hair plays a vital role in protecting the scalp as well as shaping a personal image and self-confidence. However, hair loss (alopecia) is a common and concerning global issue affecting both men and women. Among the six main types of hair loss, androgenetic alopecia (AGA) is the most prevalent, accounting for up to 95% of all cases [1]. This chronic hair loss condition is related to the sensitivity of hair follicles to dihydrotestosterone (DHT), a derivative of the hormone testosterone. AGA affects approximately 80% of men and about 50% of women, becoming particularly common after menopause [2].

Although hair loss does not pose a life-threatening risk, it significantly impacts quality of life, including psychological well-being and self-confidence. Consequently, there is a substantial demand for effective treatments. Current treatment methods primarily involve medications such as minoxidil (Regaine®), a vasodilator that stimulates hair growth, and finasteride (Propecia®), an enzyme inhibitor that prevents the

conversion of testosterone to DHT. However, the effectiveness of these treatments is limited, and potential side effects have driven the search for safer and more effective alternatives. Although finasteride is approved by the United States Food and Drug Administration (FDA) for oral treatment, it is associated with sexual dysfunction and neuropsychiatric side effects, while the oral minoxidil has been linked to hypertrichosis and cardiovascular system symptoms [3].

In this context, acetyl tetrapeptide-3 (ATP3), a synthetic peptide with the sequence Ac-KGHK-NH_2, has emerged as a promising active ingredient. ATP3 not only enhances the adhesion of hair follicles to the scalp but also promotes the development of new hair follicles. Both *in vitro* and *ex vivo* studies have demonstrated its mechanism of action. Nanoliposomes comprised of copper peptide, acetyl tetrapeptide-3, and myristoyl pentapeptide-4 have been shown to promote hair growth by enhancing the proliferation of human dermal papilla cells as well as their secretion of collagen III, vascular endothelial growth factor, and basic fibroblast growth factor [4]. Moreover, the clinical efficacy of this peptide has been validated in human trials. The study evaluated a combination of herbal extracts, including biochanin A, acetyl tetrapeptide-3, and ginseng extract, compared with a 3% minoxidil solution for treating AGA [5]. After 24 weeks, the study found comparable efficacy between the two methods. However, no adverse local reactions were observed with the herbal extract formulation, while one participant developed scalp eczema after using minoxidil. With its highly stable three-dimensional structure, ATP3 has been incorporated into commercial products and garnered significant attention from the cosmetics industry.

The solid-phase peptide synthesis (SPPS) technique is widely used in both research and industry for the efficient peptide production, even for complex sequences. By anchoring the growing peptide chain to an insoluble resin, SPPS enables precise control over reaction conditions, facilitates the incorporation of modified or non-natural amino acids to enhance stability and bioactivity [6]. In a previous study, ATP3 was synthesized via SPPS with a purity of ≥90% as determined by HPLC analysis [7]. This research aims to optimize the total synthesis of ATP3 through SPPS, ensuring a stable, high-purity raw material for hair loss treatment applications. Earlier studies explored peptide synthesis in an aqueous solvent using Smoc as a water-soluble protecting group and EDC.HCl as an activating agent [8]. However, the requirement for Smoc preparation extended the synthesis time and increased costs. To overcome these limitations, this study employs COMU as the activating agent for peptide bond formation, recognized for its safety, high activation efficiency, and non-explosive nature due to the absence of a benzotriazole group. COMU also offers good solubility, stability, and ease of byproduct removal, while its combination with DIPEA enhances catalytic efficiency [9]. The successful synthesis of ATP3 not only meets the market demand for effective cosmetic solutions but also advances treatment options, opening new avenues in hair and scalp care.

2. Content

2.1. Experiments

2.1.1. Materials

The Fmoc-protecting *L*-amino acids were used in the synthesis, including Fmoc-Gly-OH, Fmoc-His(Trt)-OH, and Fmoc-Lys(Boc)-OH, with a purity of >98%, together with the coupling agent COMU (*N*-[1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy) dimethylamino (morpholino) uronium hexafluorophosphate) also with a purity of >98%, which were all purchased from Angene, China. The solid support Rink Amide 4-methylbenzhydrylamine (MBHA) resin (100-200 mesh, 98%) was procured from AK Chemicals, USA. The solvents *N*,*N*-diisopropylethylamine (DIPEA, 99%), acetic anhydride (99%), *N*-methyl-2-pyrrolidone (NMP, 99%), dimethylformamide (DMF, 99%), dichloromethane (DCM, 99.5%), trifluoroacetic acid (TFA, 99%) and HPLC-grade acetonitrile (ACN, 99.8%) were obtained from Daejung, South Korea. Triisopropylsilane (TIS, 98%) was purchased from Sigma-Aldrich, Germany.

The high-performance liquid chromatography (HPLC) system Agilent 1260 and chromatography columns were purchased from Agilent, USA. The LC-MS/MS mass spectrometry system, Shimadzu 8040, was obtained from Shimadzu, Japan. Additional equipment, including centrifuges, shakers, a vacuum manifold reaction station, a vacuum pump, an ultrasonic bath, and other consumables such as polypropylene tubes and pipette tips, was procured from China.

2.1.2. Peptide synthesis and purification

**** Principle of Solid-Phase Peptide Synthesis***

The synthesis and purification process of acetyl tetrapeptide-3 followed a similar protocol to those previously published for acetyl hexapeptide-8 and pentapeptide-3 by the research group [10,11]. The process includes the following steps:

i. Deprotection of the protected amine group from the Rink Amide MBHA resin by removing the Fmoc protecting group.

ii. Coupling of the amino acid to the resin through a reaction between the carboxyl group of the amino acid and the amino group on the resin.

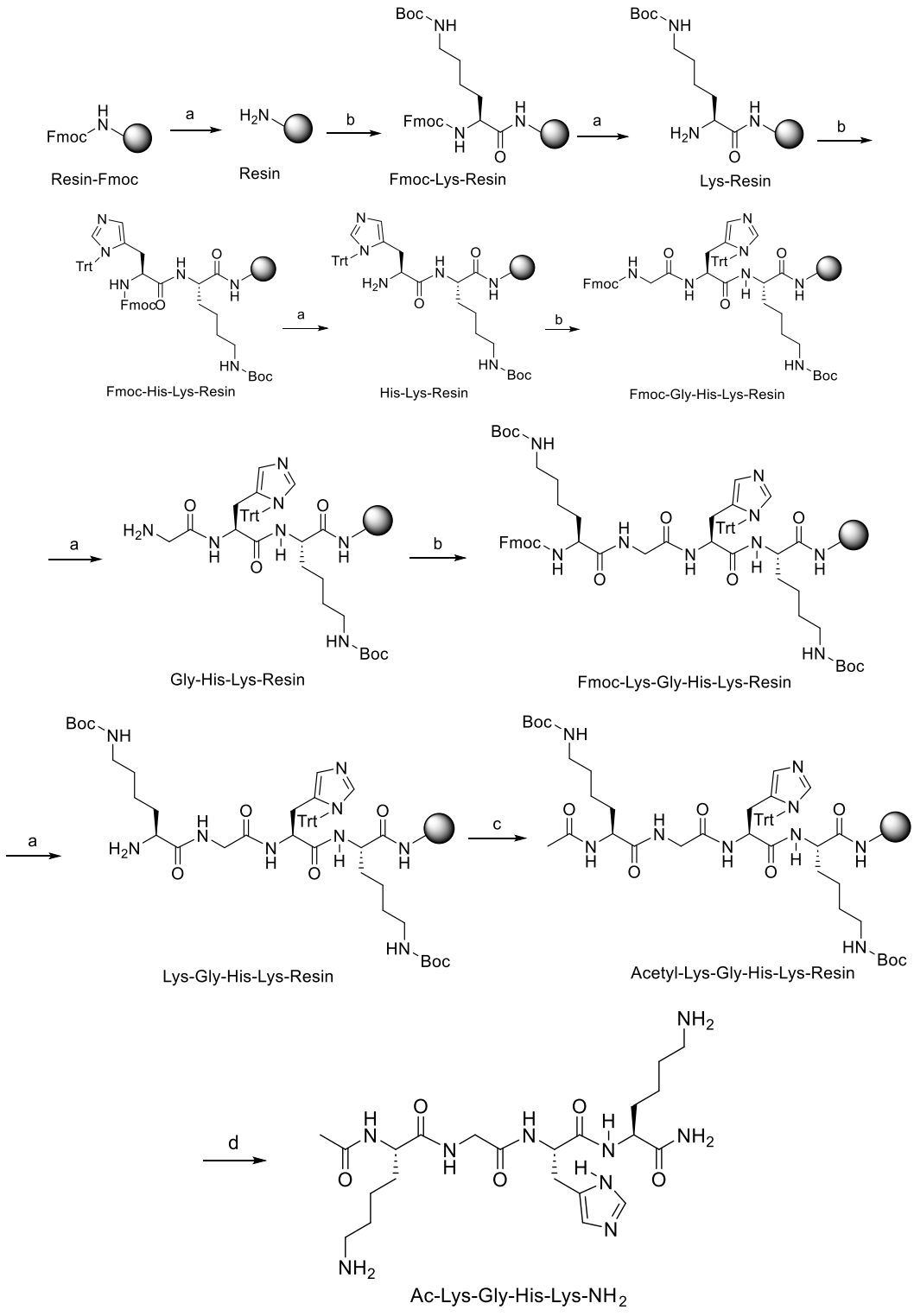
iii. Removal of the Fmoc protecting group from the amino acid attached to the resin.

iv. Sequential coupling of amino acids via amide bond formation, followed by deprotection of Fmoc groups, repeating step iii until all amino acids are incorporated.

v. Final deprotection of the Fmoc group, followed by acetylation of the tetrapeptide chain attached to the resin using a mixture of acetic anhydride, DIPEA, and NMP in a ratio of 4.25:15.75:80.

vi. Cleavage of the peptide from the resin, removal of the protecting groups from lysine and histidine, and subsequent purification to obtain pure acetyl tetrapeptide-3.

The synthesis scheme for acetyl tetrapeptide-3 is illustrated in Scheme 1.



Scheme 1. Synthesis process of acetyl tetrapeptide-3. Conditions and reagents: (a) piperidine/DMF; (b) Fmoc- amino acid, COMU, DIPEA, DMF; (c) acetic anhydride: DIPEA:NMP (4.25:15.75:80); (d) TFA:TIS:H_2O (95:2.5:2.5)

**** The acetyl tetrapeptide-3 synthesis process***

The resin (60 µmol) was soaked in DMF for 10 minutes before use. The Fmoc protecting group was removed using 20% piperidine in DMF. Amino acids were sequentially coupled using COMU as a coupling agent (resin/amino acid/COMU: 1/5/5) in excess amounts of DIPEA. After each amino acid coupling or Fmoc deprotection step, the resin was washed sequentially with DCM and DMF. The final step involved the acetylation of the peptide chain. The quantities of the materials used are detailed in Table 1.

Table 1. Chemicals for the synthesis of acetyl tetrapeptide-3

Chemicals	**MW (g/mol)**	**Quantity**	**Weight (mg)/ Volume (µL)**		**The volume of DMF (mL)**	
			Each time	***Total***	***Each time***	***Total***
Resin	732.86	1	174 mg	174 mg	-	-
Fmoc-Gly-OH	297.30	1	89 mg	89 mg	0.6	0.6
Fmoc-His (Trt)-OH	619.70	1	186 mg	186 mg	0.6	0.6
Fmoc-Lys (Boc)-OH	353.40	2	141 mg	282 mg	0.6	1.2
COMU	428.27	4	122 mg	488 mg	0.6	2.4
DIPEA	129.25	4	135 µL	540 µL	-	-
Acetic anhydride	102.09	1	170 µL	170 µL	-	-
NMP	99.13	1	9 µL	9 µL	-	-
DIPEA	129.25	1	33.47 µL	33.47 µL	-	-

(- : not used)

The reaction was monitored using the Kaiser test, which involves three reagents: solution A (40.0 g phenol dissolved in 10 mL ethanol), solution B (1.0 g ninhydrin in 20 mL ethanol), and solution C (16.5 mg KCN dissolved in 25 mL H_2O, then diluted 50 times with pyridine). To conduct the test, a small amount of resin was washed thoroughly after the reaction with DMF, DCM, and methanol (MeOH) and then placed in a test tube. Two drops of each solution A, B, and C were added, followed by heating at 110-115°C for 2 minutes. If the resin beads do not change colour and the solution remains yellow, free primary amines are absent. If the resin beads or solution develop a light blue color, free primary amines are present [12].

2.2. Purification and purity check

After removing the Fmoc protecting group, the resin was washed multiple times with DCM and then dried overnight. The resin was subsequently treated with a mixture of TFA/TIS/H_2O (95/2.5/2.5) for 2 hours to cleave the peptide from the resin and remove other protecting groups, followed by drying overnight. The peptide was then dissolved in an acetonitrile/water mixture (20/80) and filtered to remove the resin. Further purified was performed using an HPLC system with a Zorbax C18 column (Agilent, 5 µm, 9.4 x 250 mm) under the following gradient program: 5-15% B over 10 minutes, 15-100% B over 3 minutes, 100% B for 5 minutes, 100-5% B over 2 minutes, and 5% B for 1 minute. The eluents were A: 0.1% TFA in H_2O and B: 0.1% TFA in ACN, with a flow rate of 3 mL/min. The purified peptide was analyzed before and after purification using the following HPLC gradient program: 0-30% B over 8 minutes, 30-100% B over 3 minutes, 100% B for 1 minute, 100-0% B over 2 minutes, and 0% B for 2 minutes. The eluents remained the same as above, with a flow rate of 1 mL/min. Peptide characterization was performed using the LC-MS system (Shimadzu LC-MS-2020) with the gradient program: 30-80% B over 2 minutes, 80-30% B over 2 minutes. The eluents were A: 0.1% formic acid in H_2O and B: 0.1% formic acid in ACN, with a flow rate of 1 mL/min and a mass range of 400-2000 Daltons. Acetyl tetrapeptide-3 was identified based on its characteristic amide bond with maximum absorbance at 220 nm [9].

2.3. Results and discussion

During the sequential coupling of amino acids in the synthesis of acetyl tetrapeptide-3, the reactions were monitored using the Kaiser test. After each coupling, a few resin beads were tested with the Kaiser reagent. The resulting mixture appeared light yellow, indicating that the Fmoc-protecting amino acids had been successfully incorporated into the chain and the previously free amino group was no longer present. Similarly, the peptide sample was tested after the Fmoc group was removed using 20% piperidine inDMF. The resulting mixture turned dark blue, demonstrating that the Fmoc protecting group had been cleaved from the peptide chain, liberating a free amino group. Upon completing the synthesis, the crude product was purified using an HPLC system, as described below.

2.3.1. Peptide purification by HPLC system

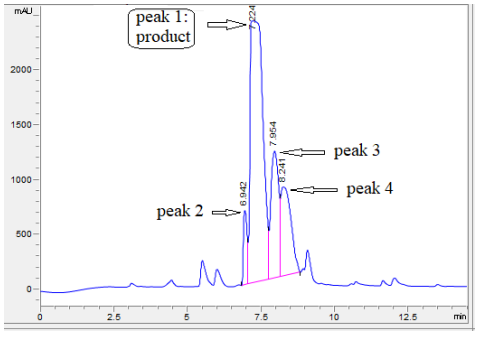
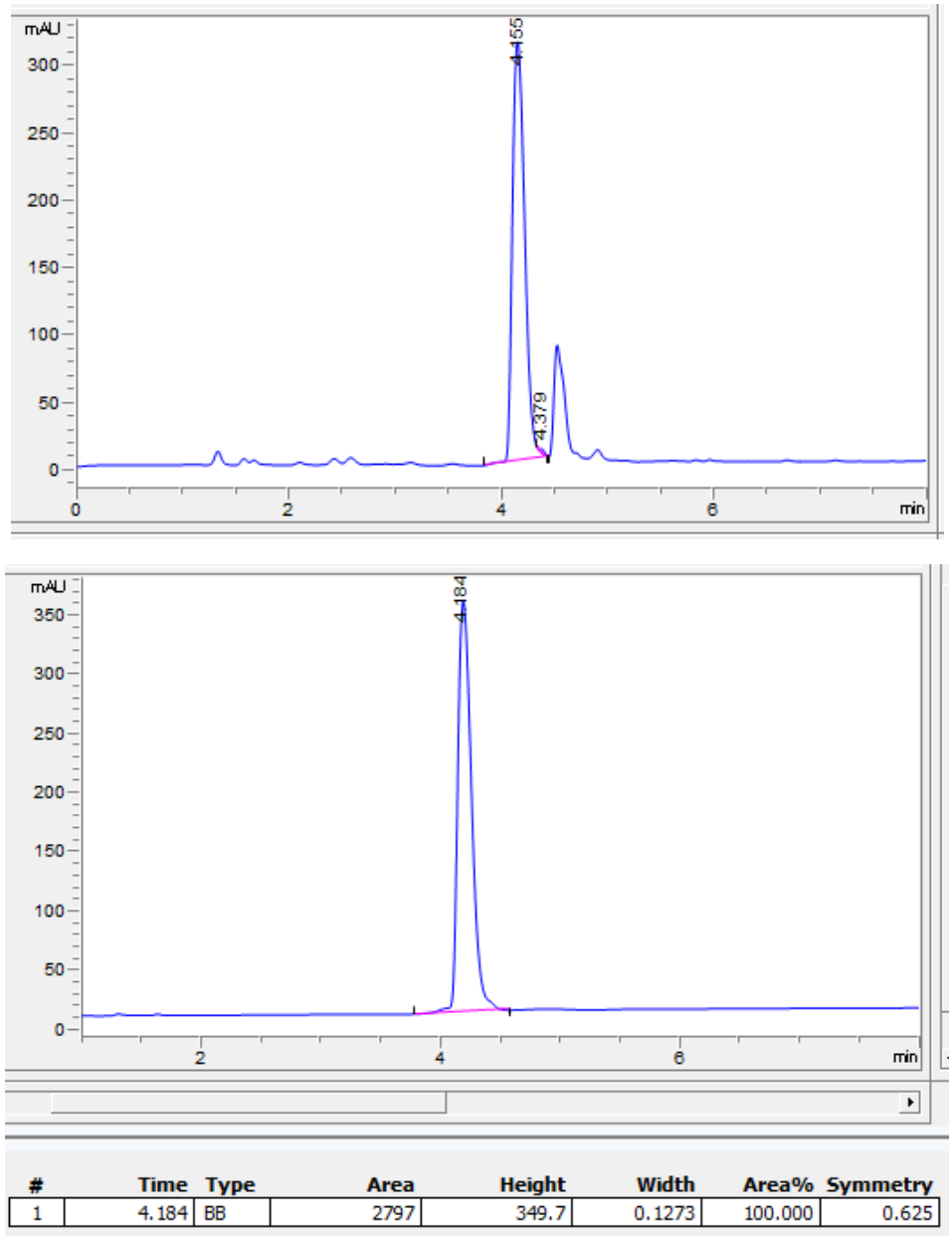


Figure 1. The preliminary chromatogram of the purification of acetyl tetrapeptide-3. Among the four peaks 1, 2, 3 and 4, only peak 1 had the molecular mass consistent with the theoretical value of acetyl tetrapeptide-3

The chromatogram illustrating the purification process is shown in Figure 1. The product peak was identified at the retention time of 7.024 minutes, and its molecular weight determined by the LC-MS system, matched the theoretical value. Subsequently, the obtained product was re-analyzed, and the results showed a single clean peak on the HPLC with the retention time matching that of the major peak in the chromatogram of the crude acetyl tetrapeptide-3 sample before purification. The chromatograms of the crude acetyl tetrapeptide-3 sample and the purified product, used for purity assessment, are presented in Figure 2.



#	Time	Type	Area	Height	Width	Area%	Symmetry
1	4.184	BB	2797	349.7	0.1273	100.000	0.625

Figure 2. Analysis of the acetyl tetrapeptide-3 sample before purification (upper image) and after purification (lower image)

The HPLC data show that the product has a retention time of 4.184 minutes, which corresponds to the 4.155-minute retention time of the main peak in the chromatogram of the crude sample before purification. Purity is defined as the percentage of the product peak area relative to the total peak area on the chromatogram (excluding system noise peaks). Accordingly, the product purity, determined by HPLC, is nearly 100%.

2.3.2. LC-MS data

After purification and purity assessment by HPLC, the acetyl tetrapeptide-3 sample was subjected to the LC-MS analysis. The mass spectrum of acetyl tetrapeptide-3 is shown in Figure 3.

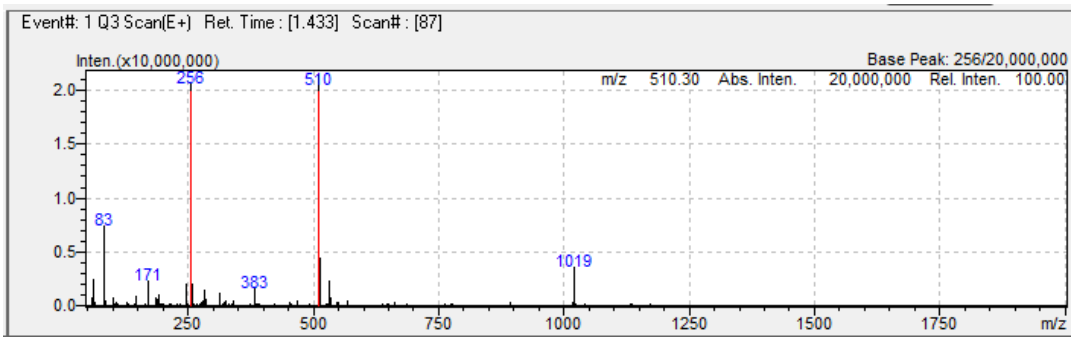


Figure 3. The mass spectrum of acetyl tetrapeptide-3 after purification

The results presented in Table 2 show that the obtained product is acetyl tetrapeptide-3, with the measured molecular weight matching the theoretical value.

Table 2. LC-MS data of Acetyl Tetrapeptide -3

Product	Chemical formula	m/z	Calculated	Found
Acetyl tetrapeptide-3	$C_{22}H_{39}N_9O_5$	$[M+1H]^+/1$	510.3153	510.30
		$[M+2H]^{2+}/2$	255.6620	256.00
		$[M+3H]^{3+}/3$	170.7770	171.00

2.3.3. Solvent removal

The acetyl tetrapeptide-3 product, after purification, was subjected to solvent removal using a rotary evaporator and then lyophilized. The obtained product was in the form of white, odorless crystals, as shown in Figure 4 below. The final yield was 17.2 mg of acetyl tetrapeptide-3, with a process efficiency of 56.29%.

Figure 4. Acetyl tetrapeptide-3 product

3. Conclusions

Acetyl tetrapeptide-3 was successfully synthesized using solid-phase peptide synthesis and subsequently purified by HPLC. The final product achieved 100% purity, as confirmed by HPLC analysis, with an overall process yield of about 56%. These results provide a foundation for the development of effective cosmetic products for hair loss treatment and scalp care, utilizing acetyl tetrapeptide-3 as a key ingredient.

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