[1-Desamino,7-lysine,8-arginine]vasotocin: Attachment of Reporter Groups and Affinity Ligands through the Lysine Side Chain

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The chemically reactive groups of [8-arginine]vasotocin (AVT) are the α-amino group in position 1, the phenolic hydroxyl group of the tyrosyl residue in position 2, and the side-chain functional group of the basic amino acid residue in position 8. Acylation or alkylation of any of these chemically reactive groups yields hormone analogues with sharply diminished biological activities in most assay systems. Since none of the chemically reactive groups in the native AVT (or other neurohypophyseal hormone) sequences is a suitable chemical port for acylation with affinity ligands and reporter groups, we have undertaken the rational design of AVT analogues in which a residue capable of being acylated has been incorporated into points of the AVT structure where structural modifications are expected to have as little effect as possible on biological activity. Empirical structure–activity relationships among neurohypophyseal hormone analogues as well as conformational models in solution and in the crystalline state suggest that positions 4 and 7 are likely points for the introduction of acylation ports. We have previously synthesized an analogue of [1-desamino]AVT (dAVT) with a lysyl residue in position 4 ([4-lysine]dAVT) and demonstrated that acylation of the side chain of this residue yields useful reporter and photoaffinity analogues. We now report the synthesis of the corresponding analogue with a lysyl residue in position 7 ([7-lysine]dAVT), which also yields potent acyl derivatives suitable for affinity ligands and photoaffinity ligands.

It is frequently necessary to introduce chromophores, fluorophores, radiolabels, affinity-chromatography handles, or photoaffinity ligands into peptide hormone analogues to probe subtle characteristics of their biological activities and conformational states. This is most conveniently accomplished if these prosthetic groups can be attached to the hormone molecule through one of its own chemically reactive functional groups. The α-amino group at the N-terminus and the phenolic group of the tyrosyl residue in position 2 are suitably reactive points of the neurohypophyseal hormone molecules, as are the side-chain functional groups of the basic amino acid residues that occur naturally in the vasotocins and vasopressins at position 8. Unfortunately, alkylation or acylation of any of these naturally reactive groups in the neurohypophyseal hormones sharply decreases biological activity in most target systems. An investigator needing neurohypophyseal hormone analogues containing reporter groups and affinity handles is, therefore, faced with the choice of incorporating these groups into the side chains of amino acid residues during the synthesis of each analogue or of designing a parent peptide sequence containing a convenient chemical port to which "working" groups can be attached later.

We required a hormone analogue that would have a convenient chemical port for attachment of ligands and reporter groups and, after attachment, be biologically active. From consideration first of empirical structure–activity relationships among natural and synthetic hormone analogues[1-3] and second of the conformation (three-dimensional geometry) of the neurohypophyseal hormone molecules in solution[4-7] and the crystalline state,[8,9] we decided that chemical attachment points might be accommodated at positions 4 and 7. In recent studies, we have synthesized [1-desamino,4-lysine,8-arginine]vasotocin and shown that acylation of the side chain of the lysyl residue in position 4 of this analogue can yield potent reporter and photoaffinity analogues.[10] We now report the solid-phase synthesis of an analogue of [1-desamino,8-arginine]vasotocin (dAVT[11]) that has a lysyl residue in position 7. This analogue, [7-lysine]dAVT (1), manifests approximately 29 units/mg of rat antidiuretic activity and neither agonist nor antagonist activity in the rat pressor assay. It is approximately half as potent as [8-arginine]-vasopressin (AVP) in the toad bladder hydroosmotic assay. The side-chain amino group of the lysyl residue in position 7 was readily acylated with *d*-biotin *p*-nitrophenyl ester

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(11) All optically active amino acids are of the L configuration. All nomenclature is in accordance with the recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Int. J. Peptide Protein Res.* **1984**, *24*, 19). Abbreviations used are: AVP, [8-arginine]vasopressin, Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2; AVT, [8-arginine]vasotocin, Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH_2; Boc, *tert*-butoxycarbonyl; 2-ClZ, 2-chlorobenzyloxycarbonyl; dAVT, [1-desamino, 8-arginine]vasotocin, Mpa-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH_2; DMF, dimethylformamide; ED_{50}, effective dose for 50% of maximal response, concentration of peptide that yields half-maximal response; HPLC, high-performance liquid chromatography; Mpa, β-mercaptopropionic acid; Meb, 4-methylbenzyl; pA_2, the negative of the logarithm of the molar concentration of antagonist required to double the concentration of agonist necessary for half-maximal response; SD, standard deviation; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Tos, tosyl.

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Table I. Rat Antidiuretic and Pressor Activities[a] of [8-Arginine]vasotocin (AVT), [1-Desamino]AVT (dAVT), and Analogues of dAVT with Modifications in Position 7

peptide	antidiuretic	pressor
AVT[b]	231 ± 30	160 ± 4
dAVT[c]	890 ± 100	256 ± 6
[7-lysine]dAVT (1)	29 ± 7	0
[7-lysine(*d*-biotinyl)]dAVT (2)	4.3 ± 1.1	0
[7-lysine(4-azidobenzoyl)]dAVT (3)	19.7 ± 4.5	*d*

[a] Units per milligram; mean ± SD. [b] Reference 28. [c] Reference 29. [d] This analogue is an antagonist in the rat pressor assay; it has a pA_2 value of 6.6.

Table II. Toad Bladder Hydroosmotic Activity of [8-Arginine]vasopressin (AVP) and Analogues of [1-Desamino,8-arginine]vasotocin (dAVT) with Modifications in Position 7

peptide	ED_{50},[a] M	n[b]	p[c]
AVP	$(9.5 \pm 5.8) \times 10^{-9}$	4	
[7-lysine]dAVT (1)	$(2.2 \pm 1.3) \times 10^{-8}$	5	ns
[7-lysine(*d*-biotinyl)]dAVT (2)	$(2.1 \pm 0.5) \times 10^{-7}$	5	<0.001

[a] ED_{50}, effective dose for 50% of maximal response; mean ± SD. [b] n, number of bladders. [c] p, probability; Student's t test; comparison to bladders challenged with AVP.

to yield an analogue, [7-lysine(*d*-biotinyl)]dAVT (2), that manifests 4.3 units/mg of antidiuretic activity and gives a full hydroosmotic response in the toad bladder at approximately 10^{-7} M. This analogue was developed for eventual use in localization studies with ferritin-labeled avidin[12] and in affinity columns based on biotin–avidin interactions.[13-15] The side-chain amino group of analogue 1 has also been acylated with *N*-succinimidyl 4-azidobenzoate to yield a potent dAVT analogue, [7-lysine(4-azidobenzoyl)]dAVT (3), which is being evaluated as a promising photoaffinity ligand for the specific labeling of vasotocin receptors in the toad urinary bladder.[16] An interesting and unexpected byproduct of this work is the finding that this 4-azidobenzoyl derivative is a moderately effective antagonist of AVP in the rat pressor assay.[16] To our knowledge, this is the only example of an agonist (dAVT) in the rat pressor assay being converted to an antagonist solely on the basis of a structural modification in position 7.

Results

The biological activities of AVT, dAVT, the dAVT analogue 1 and its *d*-biotinyl derivative 2 and 4-azidobenzoyl derivative 3 in the rat antidiuretic and rat pressor assays are shown in Table I. The results of the toad bladder hydroosmotic assay for AVP and dAVT analogues 1 and 2 are reported in Table II. Dose–response relationships in the toad bladder assay for these various compounds are depicted in Figure 1. Incubation of toad bladders in Ringer's solution containing avidin completely abolished the response to 6×10^{-7} M *d*-biotinyl peptide (2), although the same bladders subsequently responded normally to a challenge with 8×10^{-8} M AVP.

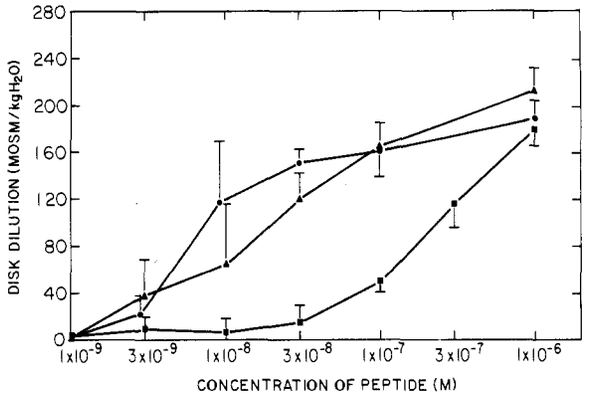

Figure 1. Hydroosmotic response of toad bladder to various concentrations of arginine vasopressin (●), analogue 1 ([7-lysine]dAVT) (▲), and analogue 2 ([7-lysine(*d*-biotinyl)]dAVT) (■). For number of experiments, n, see Table II.

Discussion

dAVT analogue 1 is, in chemical terms, a very convenient "foundation" analogue for attachment of reporter groups and ligands in that it contains a single amino group that can readily be acylated. The analogue has low pressor activity, as would be expected from previous experience with substitutions in position 7 of neurohypophyseal hormone analogues.[1-3] The potency of this amino compound in the rat antidiuretic assay is moderate but nonetheless low in comparison with that of dAVT and AVT. The potency decreases further when the amino function is acylated by the *d*-biotinyl group. On the other hand, the potencies of dAVT analogue 1 in the toad bladder hydroosmotic assay is only slightly less than that of AVP. This analogue, which possesses a free amino group in position 7, is about one-half as potent as AVP, i.e., the ED_{50} was 10^{-8} M for AVP in the control bladders and 2×10^{-8} M for analogue 1 in the experimental bladders (Table II). Addition of the *d*-biotinyl group to the side chain of the residue in position 7 decreased affinity of the molecule for hydroosmotic receptors by a factor of 10, but this compound still yielded the same maximal water permeability response as did AVP (see Figure 1). Although this analogue has decreased potency, it still compares favorably in potency with other reporter-group or affinity-ligand substitutions that have been made in other analogues of neurohypophyseal hormones and tested in this system.[17,18] Indeed, the affinity of the *d*-biotinyl analogue 2 for hydroosmotic receptors is sufficiently high to ensure reasonable specificity in binding of this hormone analogue to receptor protein(s) in the toad bladder.

The effects of avidin in blocking the action of the *d*-biotinyl analogue 2 confirms the conclusion based on the analysis by high-performance liquid chromatography (HPLC) that the activity of this derivative is not a result of the unreacted precursor amino compound 1 being carried through the synthesis and co-isolated with analogue 2. It further indicates that the activity of the *d*-biotinyl derivative is intrinsic to the molecule and not a manifestation of proteolytic removal of the *d*-biotinyl group.

Conclusion

The results of these studies indicate that analogue 1 is a suitable foundation molecule to which small acyl groups

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can be easily attached on the side chain of the lysyl residue with reasonable retention of biological potency in a variety of assay systems.

Experimental Section

Solid-phase peptide synthesis was performed on a Vega Model 96 automated peptide synthesizer, and final purification was achieved by HPLC, performed isocratically on a Laboratory Data Control Model 7000 system using a Vydac 1 cm × 25 cm C-18 reversed-phase peptide and protein column. The chromatograms were developed with a flow rate of 4 mL/min and the effluent solution was monitored for UV absorption at 215 nm unless another wavelength is specifically indicated. Samples for amino acid analysis were hydrolyzed in 6 M HCl in evacuated vials heated at 110 °C for 22 h. Amino acid analyses were performed on a Beckman Model 6300 automated amino acid analyzer using ninhydrin colorimetry for detection. Thin-layer chromatography (TLC) was performed on silica gel G plates developed in one of two solvents: A, 1-butanol/pyridine/acetic acid/H_2O, 15:10:3:6 (v/v/v/v); B, ethyl acetate/acetic acid/pyridine/H_2O, 5:1:5:3 (v/v/v/v). Chlorine/starch–KI was used for detection of peptide derivatives on the silica plates.[19]

Peptide Synthesis. [1-Desamino,7-lysine,8-arginine]vasotocin (1) ([lysine-7]dAVT) was synthesized by the solid-phase method using an experimental format that has been reported previously.[20] α-Amino groups were protected by the *tert*-butoxycarbonyl (Boc) group, the side chain of the arginyl residue was protected by the tosyl (Tos) group, and the side chain of the lysyl residue was protected by the 2-chlorobenzyloxycarbonyl (2-ClZ) group. The thiol functions of the cysteinyl and β-mercaptopropionyl residues were protected by the 4-methylbenzyl (Meb) group. The phenolic function of the tyrosyl residue was unprotected. The synthesis was carried out on a benzhydrylamine-substituted copoly-styrene-1%-divinylbenzene polymer (0.51 mequiv of amine/g of resin). The peptide was cleaved from the resin with simultaneous removal of all side-chain blocking groups by treatment with anhydrous HF at 0 °C in the presence of anisole. The resultant dithiol peptide intermediate was oxidized to the intramolecular disulfide by diiodomethane in aqueous methanol at pH 8.[21] The product was isolated by chromatography on Sephadex G-15 in 50% aqueous acetic acid followed by partition chromatography on Sephadex G-25[22] in the solvent system 1-butanol/ethanol/H_2O containing 3.5% pyridine and 1.5% acetic acid (4:1:5, v/v).[20] Final purification was accomplished by chromatography on Sephadex LH-20 in methanol. dAVT analogue **1** was obtained in 34% yield based on the substitution level of the benzhydrylamine resin used in the solid-phase synthesis. It chromatographed as a single, symmetrical peak on HPLC in a solvent system composed of 6% 2-propanol in 0.1% aqueous trifluoroacetic acid. The elution time was 14 min. An acid hydrolysate yielded amino acids in the following ratio: Asp, 1.01 (1.0); Glu, 1.03 (1.0); Gly, 1.03 (1.0); Cys, 1.19 (1.0); Ile, 0.97 (1.0); Tyr, 0.99 (1.0); Lys, 1.01 (1.0); Arg, 1.00 (1.0).

[1-Desamino,7-lysine(*d*-biotinyl),8-arginine]vasotocin (**2**) ([7-lysine(*d*-biotinyl)]dAVT) was prepared by acylation of analogue **1** with *d*-biotin *p*-nitrophenyl ester (Sigma Chemical Co., St. Louis, MO). Sixty-five millgrams of the amino compound **1** was dissolved in 0.2 mL of a dimethylformamide (DMF) solution that was 1 M in *N*-methylmorpholine and 0.5 M in acetic acid (buffered DMF[23]). A 5-μL aliquot of this solution gave a strong reaction with the Kaiser ninhydrin reagent.[24] When *d*-biotin *p*-nitrophenyl ester (65 mg) was added to the solution, it began to turn yellow almost immediately. After 1 h, an aliquot of the reaction mixture gave no reaction with the Kaiser ninhydrin reagent. Acetic acid (100 μL) was added to the reaction mixture, followed by 10 mL of H_2O. The aqueous phase was filtered and then extracted twice with equal volumes of ethyl acetate. Traces of ethyl acetate were removed from the aqueous phase by rotary evaporation. A portion of the aqueous phase (60%) was applied to the C-18 HPLC column and washed with 0.1% trifluoroacetic acid (TFA). The product was eluted from the column with 7% 2-propanol in 0.1% aqueous TFA (solvent system C). Lyophilization of frozen eluate corresponding to the major peak absorbing at 254 nm yielded a white powder. HPLC of this material in either C or 16% CH_3CN in 0.1% aqueous TFA, monitored at 214 nm, showed a single major component with trace contaminants. The peptide was free of the starting material, **1**, which is well resolved from the product in both of these chromatographic systems. The trace contaminants were removed from a few milligrams of the peptide at a time by HPLC rechromatography in C (elution time, 30 min). The rechromatographed peptide migrated as a single, symmetrical peak on HPLC in 16% CH_3CN in 0.1% aqueous trifluoroacetic acid with an elution time of 16 min. dAVT analogue **2** was obtained in approximately 57% yield as a chromatographically homogeneous product, based on starting material **1**. An acid hydrolysis of this material yielded amino acids in the following ratio: Asp, 1.02 (1.0); Glu, 1.07 (1.0); Gly, 1.05 (1.0); Cys, 1.16 (1.0); Ile, 1.10 (1.0); Tyr, 0.87 (1.0); Lys, 1.04 (1.0); Arg, 0.93 (1.0).

[1-Desamino,7-lysine(4-azidobenzoyl),8-arginine]vasopressin (**3**) ([7-lysine(4-azidobenzoyl)]dAVT) was prepared[16] by acylation of analogue **1** with *N*-succinimidyl 4-azidobenzoate (Pierce Chemical Co., Rockford, IL). Analogue **1** (21.6 mg) was dissolved in 2 mL of DMF with 10.4 mg of the succinimide ester derivative. *N*-Methylmorpholine was added to the reaction mixture until a stirring rod dipped into the solution gave an apparent pH of 7.5 on a moist strip of pH paper. The solution was stirred overnight in the dark. The reaction mixture was evaporated to dryness and the residue was chromatographed on a 2.5 × 150 cm bed of Sephadex G-15 in 0.2 M acetic acid. The effluent solution was monitored at 254 nm, and 7-mL fractions were collected. The product, which eluted in fractions 122–140, was recovered by lyophilization to yield 15.6 mg of white solid. The peptide showed a single spot (R_f 0.54 in solvent A and 0.92 in solvent B) by TLC. It exhibited absorption maxima at 270 nm in the ultraviolet and at 2120 nm in the infrared region, which are characteristic of aryl azido compounds.

The compound eluted as a single symmetrical peak with a retention time of 20 min in HPLC analysis in 15% 2-propanol in 0.1% aqueous trifluoroacetic acid.

Biological activity was determined in the rat antidiuretic assay, the rat pressor assay, and the toad bladder hydroosmotic assay.

Rat Antidiuretic Assays. Antidiuretic activities were assayed on water-loaded, ethanol-anesthetized rats, according to the method of Sawyer.[25]

Rat Pressor Assays. Pressor activities were assayed on urethane-anesthetized, phenoxybenzamine-treated rats, as described by Dekanski.[26]

Toad Bladder Hydroosmotic Assays. Toads of the species *Bufo marinus* (Dominican Republic) were doubly pithed, and their urinary bladders excised. The bladders were suspended as sacs on the ends of glass tubing with their serosal sides out. Bladders were filled with Ringer's fluid diluted to $^1/_5$ normal strength and suspended in Ringer's solution to which NaCl had been added to raise fluid osmolality to approximately 290 mOsm/kg H_2O. The action of peptides to increase bladder permeability to water was measured by an osmometric method reported in detail elsewhere.[27] In brief, bladders were suspended in humidified air and paper disks containing 8 μL of hypertonic Ringer's solution with or without various concentrations of peptides were applied to the outer surface of the bladder wall. After 15 min, the disks were removed from the bladder wall with fine forceps and immediately placed in the chamber of a vapor pressure osmometer (Model

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5100C, Wescor, Inc., Logan, UT). The dilution of the disk fluid below controls without peptide was taken as a measure of osmotic water flow from the mucosal bath into the serosal surface disk. For these studies AVP (Pitressin, Parke-Davis, Morris Plains, NJ) was used as the reference standard.

The Ringer's solution employed in these experiments was composed of the following solutes in mmol/L: NaCl, 110; KCl, 3.5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 5.5; tris(hydroxymethyl)aminomethane hydrochloride, 10; pH, 7.5; osmolality, 236 mOsm/kg H_2O.

Toad Bladder Hydroosmotic Assay in the Presence of Avidin. Toad bladders were preincubated for 15 min in Ringer's solution containing 4 mg/mL avidin (Sigma Chemical Co.). Analogue 1 and AVP were assayed in the continued presence of avidin.

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Binary Drugs: Conjugates of Purines and a Peptide That Bind to Both Adenosine and Substance P Receptors

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A "functionalized congener" approach to adenosine receptor antagonists has provided a means to synthesize highly potent peptide conjugates of 1,3-dialkylxanthines. The antagonist XAC, such a functionalized xanthine amine congener, has been attached to a segment derived from the neurotransmitter peptide substance P (SP) to form a binary drug that binds to both receptors with K_i values of 35 nM (central A_1-adenosine) and 300 nM (striatal SP). Coupling of the functionalized adenosine agonist N^6-[*p*-(carboxymethyl)phenyl]adenosine to an SP C-terminal peptide also resulted in a binary drug that binds to both receptors. The demonstration that the biochemical properties of two unrelated drugs, both of which act through binding at extracellular receptors, may be combined in the same molecule suggests a novel strategy for drug design. In principle, a combined effect of the two different substances that produce the same final effect (e.g., hypotension by adenosine agonists and by SP analogues) might occur in vivo. Adenosine analogues have analgesic properties, and the binary drug derived from substance P and adenosine agonists or antagonists might provide useful tools for probing interrelationships of SP pathways and sites for the antinociceptive action of adenosine.

A "functionalized congener" approach to adenosine receptor antagonists[1,2] has resulted in highly potent 1,3-dialkylxanthines having amino and carboxylic acid functionalized chains. These functional groups have been coupled covalently to amino acids and dipeptides. The resulting amino acid and oligopeptide conjugates have full, and in some cases enhanced, biological activity. In our previous work[2] we used nonhormonal peptide sequences to alter the physicochemical properties of the drugs. We now report the attachment of a xanthine to a segment derived from a putative neurotransmitter peptide, substance P (SP) (reviews, ref 3,4), to form a binary drug 1 (Figure 1). This drug binds both to adenosine receptors and to substance P receptors with affinity comparable to that of each component at the appropriate receptor. In addition, coupling of the adenosine agonist N^6-[*p*-(carboxymethyl)phenyl]adenosine,[5] **2a**, to substance P segment 7–11 produced the binary drug **3**, which also binds to both receptors.

We have demonstrated a potentially general strategy via "functionalized congeners" for the synthesis of binary drugs that combine pharmacological properties of both elements. The choice of combination of adenosine receptor ligands and substance P in target molecules stems from the peripheral hypotensive effect elicited by agonists for both receptors.[6,7] Moreover, since both receptors mediate central nociceptive effects,[8,9] the binary conjugates may prove to be useful tools in probing neuronal pathways

Table I.[a] Potencies of the Binary Drugs and Various Truncated Derivatives and Intermediates at A_1-Adenosine Receptors and at Substance P Receptors

	K_i, nM	
	A_1-adenosine receptors	SP receptors
substance P analogues		
H-Phe-Phe-Gly-Leu-Met-NH_2 (**8a**)	>30 000	>10 000
Ac-Phe-Phe-Gly-Leu-Met-NH_2 (**11**)	>30 000	>10 000
H-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (**8b**)		450
H-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (**8c**)		180
substance P	>30 000	1.1
xanthine derivatives		
XAC (**4a**)	1.2 ± 0.3	>10 000
H-Gly-XAC (**4b**)	2.1 ± 0.1	>10 000
H-Asn-XAC (**4c**)	4.3 ± 0.2	
H-Asp(XAC)-Phe-Phe-Gly-NH_2 (**10**)	30 ± 6.0	>10 000
H-Asp(XAC)-Phe-Phe-Gly-Leu-Met-NH_2 (**1**)	35 ± 3.4	300
adenosine derivatives		
N^6-$C_6H_4CH_2COOH$ (**2a**)	210 ± 57	
N^6-$C_6H_4CH_2CONHCH_3$ (**2b**)	16 ± 10	>10 000
N^6-$C_6H_4CH_2CO$-Phe-Phe-Gly-Leu-Met-NH_2 (**3**)	16 ± 0.9	2000

[a] The complete sequence of substance P is Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2.

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