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Stepwise synthesis of oligonucleotide-peptide conjugates containing guanidinium and lipophilic groups in their 3'-termini

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Abstract. Two different series of oligonucleotide-peptide conjugates have been efficiently synthesized by stepwise solid-phase synthesis. First, oligonucleotides and oligonucleotide phosphorothiotes containing polar groups at the 3'-termini, such as amine and guanidinium groups were prepared. ODNs conjugates carrying several lysine residues were obtained directly from Fmoc deprotection whereas ODN conjugates with guanidinium groups were obtained by post-synthetic guanidinylation. The second family contains different urea moieties that were achieved by standard protocols. All products were fully characterized by reversed phase HPLC and MALDI-TOF mass spectrometry yielding satisfactory results. Oligonucleotide phosphorothioate conjugates were evaluated as potential antisense oligonucleotides in the inhibition of the luciferase gene.

The use of modified oligonucleotides (ODNs) as potential therapeutics has emerged in the last few years with the antisense and gene-silencing technologies as the most important applications.[1] However, the low stability of these ODNs against exonucleases and endonucleases along with their poor cellular uptake properties (as a result of oligonucleotide size and the repulsion of the negatively charged phosphate backbone) have turned into a real bottleneck in the use of these compounds as clinical drugs.[2] Many attempts to change these limitations have been made including the substitution of the aforementioned phosphate internucleotide bond with phosphorothioate groups[3] or the conjugation of cationic lipophilic carriers,[4] polymers or nanoparticles5 with ODNs thereby improving their ability to transverse cell membranes.

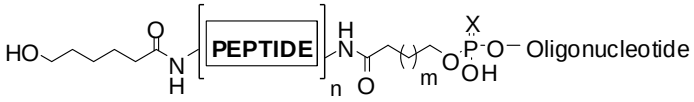
Recently, the use of synthetic transporters mimicking cell-penetrating peptides $(CPPs)_6$ has become a powerful method for transporting materials across the cell membrane,[7] especially nucleic acids (e.g. short interfering RNAs (siRNAs),[8] aptamers[9] and antisense oligonucleotides (ASO)[10]). Taking this into account, our research group has become interested in the preparation of ODNs carrying CPPs for gene silencing.[11] From a synthetic point of view, there are two different strategies for obtaining ODN-peptide conjugates: (1) the non-covalent approach, where amphipathic peptides can condense with DNA molecules, and (2) the covalent approach, which involves formation of a

covalent conjugate between the cargo and the peptide. These strategies have recently been covered in several reviews.[12]

In addition to the use of CPPs as internalization agents, the introduction of additional groups into the CPPs may help to increase the cellular uptake of the corresponding ODN-CPP conjugates. For instance, it is known that the guanidinium group (pka = 12.5) plays an important role in several biological processes due to its highly basic and positively charged character over a wide pH range.[13] This moiety has been incorporated onto different positions of ODN-CPP conjugates such as the nucleobase,[14] the 2'-position of the sugar ring[13c,15] and the phosphate internucleotide bond.[16] Interestingly, CPP containing guanidinium moieties (arginine octamer, R_8)[17] and oligonucleotide-arginine conjugates (such as siRNAs[18] and ASO[19] derivatives) have shown promising results in cell delivery.

However, only non-covalent synthetic approaches or solution-phase conjugations (somewhat limited due to its high cost and time consuming processes) have been reported, making total stepwise solid-phase synthesis[20] a possible alternative for obtaining such conjugates.

We have therefore explored the possibility of synthesizing ODN-CPP conjugates containing two, four and eight amine and guanidinium moieties (ODN-$(lysine)_n$ and ODN-$(homoarginine)_n$ derivatives; Figure 1) following a stepwise solid-phase approach. Moreover, we have focused on the stepwise synthesis of another family of ODN-CPP conjugates carrying different lipophilic urea moieties (e.g. aliphatic and aromatic units; Figure 1). Finally, this synthetic strategy has been used to synthesize oligonucleotide phosphorothioate derivatives designed to inhibit the Renilla luciferase gene[21] and carrying the aforementioned modifications (lysines, homoarginines and ureas). The novel oligonucleotide phosphorothioate derivatives have been evaluated in cells and their antisense activities have been compared to the respective control antisense ODN.



Peptide = Lys and HArg ; n = 1, 2, 4 and 8

where m = 0 and 2 ;

X = O, S

Figure 1. ODN-CPP conjugates synthesized by the stepwise solid-phase approach.

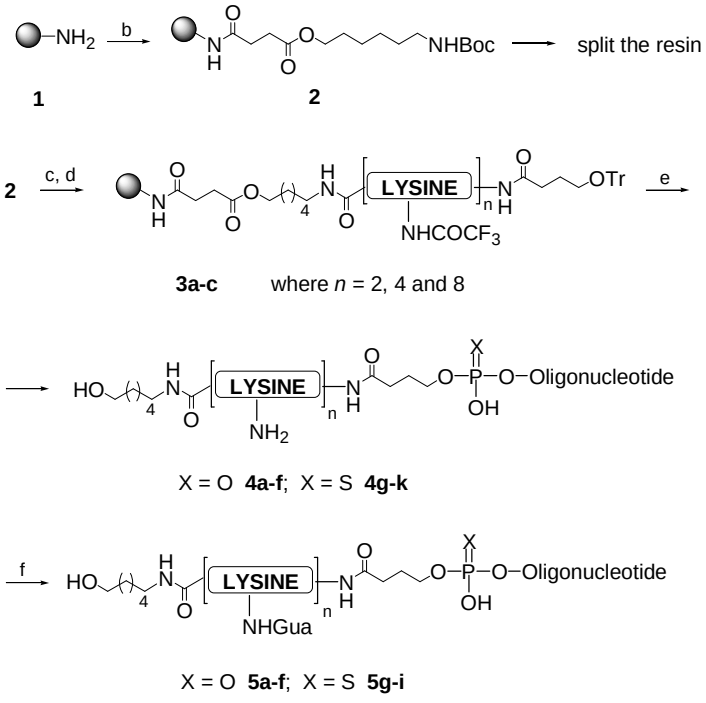
Four oligonucleotide sequences were chosen: two phosphodiester sequences (the self complementary sequence A (Dickerson-Drew dodecamer) 22 and a single strand 15-

mer of sequence B (see Table 1)) and two phosphorothioate sequences C and D for antisense studies.

Table 1. Oligodeoxynucleotide sequences

	backbone	Sequences (5'-3')
A	phosphodiester	CGCGAATTCGCG
B	phosphodiester	TAGAGGGTCCATTGC
C	phosphorothioate	AGGTCTTGTTTCCTTTGC
D	phosphorothioate	CTGTCTGACGTTCTTTGT

First, we focused on the solid-phase synthesis of oligonucleotide phosphodiester-lysine conjugates (4a-f; Figure 2). The synthesis of the conjugates 4a-f (ODN-Lysine)n (n = 2, 4 and 8) started with the assembly of the peptide sequences. Incorporation of two, four and eight lysine units in the resin was carried out using the stepwise approach[23] and standard "Boc-chemistry" protocols.



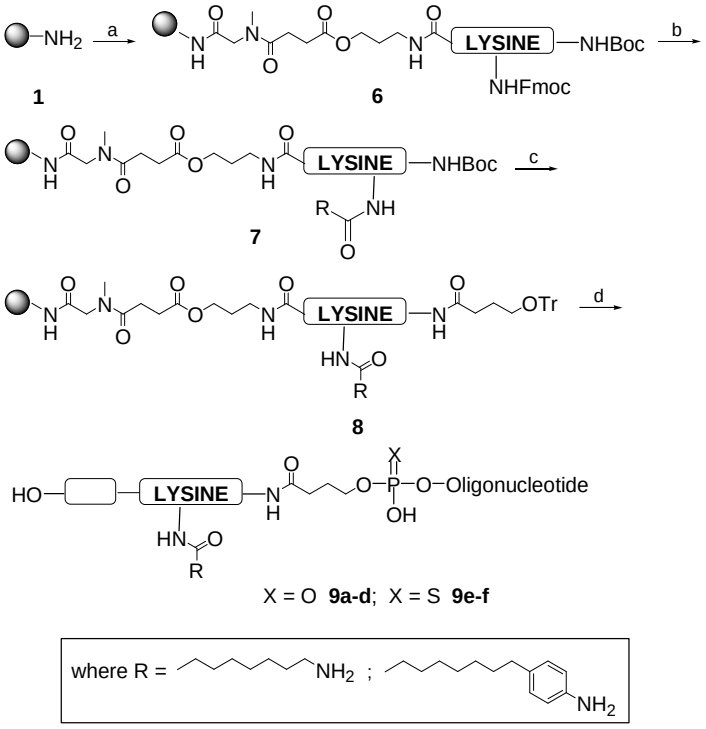
Reagents and Conditions: a. DMAP, DCM, r.t., overnight; b. spacer-1, PyBOP, DIPEA, DMF, r.t.; *Introduction of lysine residues*: c. i. DCM:TFA (2:3); ii. BocLys(tfa)OH, PyBOP, DIEA, DMF, 2h; *Introduction of OTr-spacer*: d. i. DCM:TFA (2:3); ii. spacer-OTr, PyBOP, HOBt, DMF, overnight; e. i. DNA synthesis, ii. NH_3 (32%), 55ºC, overnight; iii. NAP-10; iv. HPLC purification; v. AcOH 80%; f. *Guanidynilation reaction*: i. 4a-k; *O*-Methylisourea, NH3 (32%), 55ºC, overnight; ii. NAP-5

Figure 2. Synthesis of solid supports 3a-c, ODN-lysine conjugates (4a-k) and ODN-homoarginine conjugates (5a-i)

Commercially available poly(ethylene glycol)-polystyrene (PEG-PS, 1) (PerSeptive Biosystems, 0.19 mmol/g) was used as our starting material. Before introducing the aforementioned lysine units (from commercial available Boc-Lys-tfa-OH amino acid, Novabiochem), the solid support 2 was equally divided into three parts. Then, the respective peptides (formed by two, four and eight lysine units, respectively) were synthesized. The last Boc group was removed (under acidic conditions) after completing the peptide sequence. The resulting free amine was protected with 4-(trityloxy)butanoic acid[24] to give the respective solid supports 3a, 3b and 3c. The modified supports were then employed in the preparation of ODN strands using a DNA synthesizer. In all cases, coupling yields were around 95% by the analysis of the DMT group released in each step. After cleaving the resins with ammonia solution (32%) at 55 ºC overnight, the corresponding modified ODN-peptide conjugates in their NH2 form were purified by DMT on based protocols to give our expected lysine conjugates (ODN-Lysine) 4a–f. These conjugates were analyzed by analytical HPLC and confirmed by MALDI-TOF mass spectrometry (MS) (Table 2).

We then incorporated the guanidinium groups into the conjugates 4a-f using the post-synthetic approach (Figure 2). The guanidinylation reaction was carried out using a modified version of the method described in the literature[16a,b] (55 ºC, overnight). In all reactions, selective and quantitative guanidinylation was observed. After desalting (Sephadex G-25), the guanidinylated-ODN-CPPs (ODN-HArg) 5a-f were analyzed by analytical HPLC and confirmed by MALDI-TOF mass spectrometry (Table 2).

Having in hand ODN-lysine and ODN-homoarginine conjugates 4-5, we focused on the solid-phase synthesis of oligonucleotide-lysine-urea conjugates (Figure 3). In order to increase the lipophilic character of such conjugates, aliphatic and aromatic urea moieties were chosen. In general, ureas have been synthesized on solid-support by several strategies:[25] The most common approaches involve the reaction of a polymer-supported amine with isocyanates[26] or active carbamates.[27] Here, we follow the method described by Kennan et al., which involves the use of p-nitrophenylchloroformiate (pNPC) as activating group.[27]



Reagents and Conditions: a. i. Fmoc-Sar-OH, PyBOP, DIPEA, DMF; ii. spacer-1, PyBOP, DIPEA, DMF; iii. Boc-Lys(Fmoc)-OH, PyBOP, DIPEA, DMF; b. i. DMF:Piperidine 25%; ii. *p*NPC, DIPEA, THF:DCM (1:1); c. i. DCM: TFA (2:3); ii. spacer-OTr, PyBOP, HOBt, DIPEA, DMF; d. i. DNA synthesis; ii. NH_3 (32%), 55ºC, overnight; iv. HPLC purification; v. AcOH 80%

Figure 3. Synthesis of ODN-lysine-urea conjugates 9a-f

First, we functionalized the solid support with a Fmoc protected sarcosine (Fmoc-Sar-OH, Bachem). After deprotection of the amino group, the spacer and protected lysine (Boc-Lys-Fmoc-OH, Bachem) units were incorporated to the functionalized resin (as described above) to give compound 6 (Figure 3). After removing the Fmoc group from resin 6, two different amines were incorporated: octylamine (a) as an example of an aliphatic amine, and 4-octylaniline (b) as an aromatic amine, thereby achieving the expected ureas 7a-b attached to the solid support. Then, the appropriate spacer 2 was added, according to the literature.[23c] The resulting solid supports (8a-b) were then employed in the preparation of ODN strands using a DNA synthesizer. The same ODN sequences A and B were prepared. Coupling yields were around 95%. The solid supports were cleaved with ammonia solution (32%), desalted (Sephadex G-25) and the expected ODN-lysine-urea conjugates 9a-d were isolated, analyzed using analytical HPLC and confirmed by MALDI-TOF mass spectrometry (Table 2).

Next, the effect of the substitutions on the thermal stability of the duplex was analyzed by denaturation studies (Table 2).

Table 2. MALDI-TOF mass spectrometry and melting temperatures (ºC) data of ODN-$(lysine)_n$, ODN-$(HArg)_n$ and ODN-lysine-urea conjugates.

Comp.	Sequence	3'-modification	Tm (ΔTm)[a]	MW (calcd)	MW (found)
4a	A	$(Lys)_2$	53.0 (8.9)	4154	4157
4b	A	$(Lys)_4$	53.4 (9.3)	4415	4427
4c	A	$(Lys)_8$	56.2 (12.1)	4931	4932
4d	B	$(Lys)_2$	54.7 (0)	5117	5125
4e	B	$(Lys)_4$	55.3 (0.6)	5373	5378
4f	B	$(Lys)_8$	54.1 (-0.6)	5890	5890
4g	C	$(Lys)_2$	33.8 (3.3)	6253	6259
4h	C	$(Lys)_4$	33.5 (3.0)	6509	6511
4i	C	$(Lys)_8$	33.7 (3.2)	7022	7020
4j	D	$(Lys)_2$	n.d.	6253	6280[b]
4k	D	$(Lys)_8$	n.d.	7022	7091c
5a	A	$(Arg)_2$	62.0 (17.9)	4240	4251
5b	A	$(Arg)_4$	62.4 (18.3)	4587	4583
5c	A	$(Arg)_8$	59.3 (15.2)	5275	5274
5d	B	$(Arg)_2$	55.4 (0.7)	5203	5202
5e	B	$(Arg)_4$	55.2 (0.5)	5545	5545
5f	B	$(Arg)_8$	53.3(-1.4)	6234	6214[d]
5g	C	$(Arg)_2$	33.7 (3.2)	6339	6337
5h	C	$(Arg)_4$	35.5 (5.0)	6681	6669[d]
5i	C	$(Arg)_8$	32. 8 (2.3)	7366	5894[e]
9a	A	Urea aliph.	59.0 (14.9)	4189	4192
9b	A	Urea arom.	58.0 (13.9)	4261	4263
9c	B	Urea aliph.	52.1 (-2.6)	5146	5147
9d	B	Urea arom.	49.0 (-5.7)	5222	5223
9e[f]	C	Urea aliph.	34.2 (3.7)	6306	6309
9f[f]	C	Urea arom.	31.0 (0.5)	6377	6380
10a	A	None	44.1 (0)	n.d.	n.d.
10b	B	None	54.7 (0)	n.d.	n.d.
10c	C	None	30.5 (0)	5769	5725
10d	D	None	n.d.	5715	5720

n.d. not determined; [a]ΔTm is the difference between the melting temperature of the modified oligonucleotide minus the melting temperature of the corresponding unmodified oligonucleotide; appropriate complementary sequences are unmodified, buffer conditions 50 mM NaCl, 10 mM sodium phosphate pH 7.0; [b][M+Na^+]; [c][M+K+Na^+]; [d][M-H_20] ethe mass correspond to the removal of the whole peptide and two spacers; fthe linkage between the peptide and the peptide was 6-(trityloxy)hexanoic acid.

Compared to the unmodified ODN (10a) having a Dickerson sequence (Tm = 44.1ºC), the corresponding ODN-peptide conjugates (lysine, homoarginine and urea derivatives 4a-c, 5a-c, 9a-b, respectively) had greater duplex stability when hybridized to their ODN complement (with ΔTm in the range of 8.9ºC-12.1ºC for 4a-c, 15.2ºC-17.9ºC for 5a-c, and 13.9ºC-14.9ºC for 9a-b). The most stabilizing modifications were the homoarginines (5a-c) probably due to electrostatic interaction of the guanidinium groups with phosphates. Unexpectedly the urea modifications (9a-b) had higher Tm's than the lysine modified dodecamers (4a-c). At present we have not explanation for this behaviour, but we have observed similar results with the same dodecamer carrying other lipids.

When compared to unmodified ODN (10b) having a 15mer sequence (Tm = 54.7ºC), the corresponding conjugates had similar duplex stability (lysine and homoarginine derivatives 4d-f, 5d-f, respectively), or lower (with ΔTm -2.6ºC and -5.7 ºC for the aliphatic and aromatic ureas 9c-d, respectively). Based on these results we hypothesize that the high Tm values observed for the Dickerson derivatives (4a-c, 5a-c,

9a-b) may be due to the unique nature of self-complementary / palindromic oligonucleotide sequences.[28]

Since the above experiments showed that ODN-(lysine)n, ODN-(homoarginine)n and ODN-lysine-urea conjugates could be effectively synthesized, it was of interest to study the potential therapeutic applications of such conjugates. In particular, we evaluated the effect of the above described modifications at the 3' end of oligonucleotide strands on the ability of the corresponding conjugate derivatives to act as inhibitors of gene expression. Furthermore, we tested the ability of such conjugates to impart cell uptake.

First, lysine, homoarginine and lysine-urea residues were incorporated at the 3' end of a phosphorothioate ASO strand that targets the 21-38 site of Renilla luciferase mRNA.21 (sequence C). Following the same experimental procedures described in Figures 2 and 3, we prepared ASO-peptide conjugates containing two, four and eight lysine and homoarginine residues (4g-i and 5g-i, respectively), and one lysine unit carrying aliphatic and aromatic urea moieties (9e and 9f, respectively; see Table 2). The unmodified phosphorothioate ASO (10c) was also synthesized. Finally the scrambled phosphorothioate sequence D was prepared, unmodified (10d) and carrying 2 (4j) and 4 (4k) lysines. All ASO derivatives were analyzed by analytical HPLC and confirmed by MALDI-TOF mass spectrometry (Table 2). Some oligonucleotide phosphorothioate conjugates suffer fragmentation during MALDI-TOF spectra acquisition but fragments could be identified as the common fragmentations occur at the spacers. Compared to unmodified ASO duplex, duplexes containing ASO-lysine, ASO-homoarginine and ASO-lysine-aliphatic urea conjugates (4g-i, 5g-i, and 9e, respectively) had higher thermal stability (with ΔTm in the range of 3ºC-5ºC), whereas the duplex containing an ASO-lysine-aromatic urea conjugate (9f) had a similar Tm value (31ºC; unmodified 30.5 ºC, Table 2).

After ASO-peptide synthesis we carried out separate gene knockdown experiments in SH-SY5Y cells. In a first series of experiments, the cells were cotransfected with two luciferase plasmids (*Renilla* and firefly; target and internal control, respectively) and ASO derivatives 10c, 4g-i, 5g-i and 9e-f (60 nM) as well as the scrambled sequences (10d, 4j and 4k) by using commercial cationic liposomes (Lipofectamine 2000). Twenty-two hours after transfection, the luciferase activities of the samples were measured by using a luminometer. The results, showing *Renilla* luciferase activity normalized to firefly luciferase, are shown in Figure 4. Interestingly, lysine, homoarginine and lysine-aliphatic urea moieties did not disrupt antisense activity. ASO-lysine, ASO-homoarginine and ASO-lysine-octylurea conjugates 4g-i (two, four and eight lysine residues), 5g-i (two, four and eight homoarginine residues) and 9e, respectively, showed activities comparable to the unmodified ASO (10c) (69% knockdown of *Renilla* expression for unmodified 10c versus 61%, 57%, 65%, 64%, 57%, 66% and 55% for 4g-i, 5g-i and 9e, respectively). Only the urea carrying an aromatic group (9f) was detrimental to antisense activity (8% inhibition). However, the results obtained for the ASO-lysine (4g-i), ASO-homoarginine (5g-i) and ASO-lysine-aliphatic

urea (9e) conjugates suggest that, in principle, lysine and homoarginine conjugation does not interfere with cellular activity.

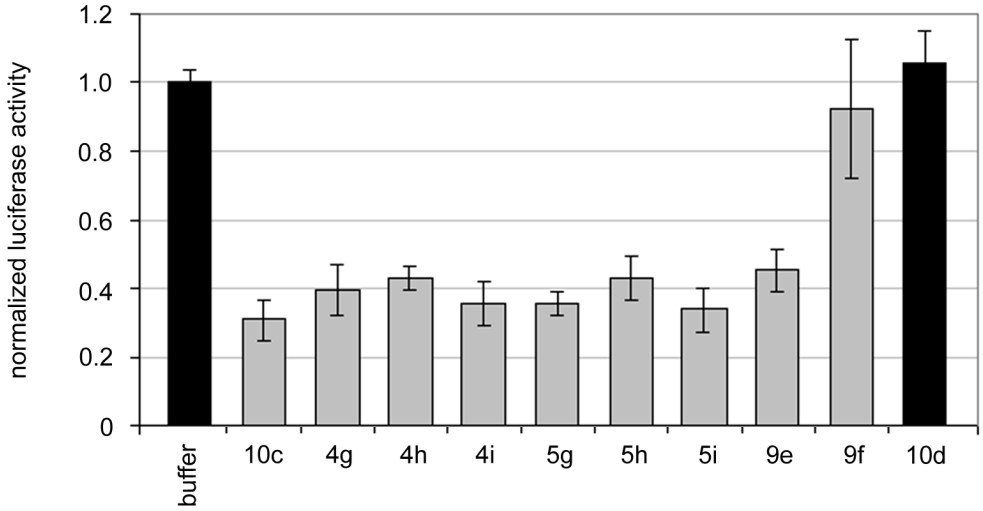


Figure 4. Plot of gene-specific silencing activities for unmodified (10c), and modified (4g-i, 5g-i and 9e-f) antisense oligonucleotides (60 nM per well) targeting the *Renilla* luciferase mRNA expressed in SH-SY5Y cells. Transfection of antisense oligonucleotides was carried out by using Lipofectamine 2000. Unmodified (10d) and lysine-modified (4j and 4k) scrambled sequences gave no *Renilla* luciferase inhibition (see above and Supplementary Figure S1)

Finally, in a second series of experiments we tested the ability of conjugates 4g-i and 5g-i to impart cell uptake. Three hours after transfection of the two luciferase plasmids, the cell medium was discarded and the cells were incubated with fresh medium and the ASO derivatives in the absence of Lipofectamine 2000 (150, 200 and 300 nM ASO-peptide). Twenty-two hours after transfection the luciferase activities were measured as described above. Although gene knockdown was significantly less efficient than that observed for ASO-peptide-lipofectamine complexes, results (see Supplementary Figure S2) showed that all the ASO-peptide conjugates tested were able to penetrate SH cells in the absence of a transfection agent (11%, 15%, 11%, 18%, 21%, 28% knockdown at 300 nM of Renilla expression for 4g-i and 5g-i, respectively, versus 1.5% for 10c). Interestingly the ASO-homoarginine octamer (5i) was able of inducing antisense activity at levels of ~28% at 300 nM.

In summary, in this work we have developed a new strategy for the stepwise synthesis of ODN-$(lysine)_n$, ODN-$(homoarginine)_n$ and ODN-lysine-urea conjugates. This method has allowed us to synthesize for first time several ODN covalently linked to the homoarginine octamer using a stepwise synthesis protocol. Antisense studies in mammalian cells suggest that lysine and homoarginine conjugation does not interfere with cellular activity. Moreover, ASO-lysine and ASO-homoarginine conjugates have been shown to impart cell uptake. These encouraging results will be followed by more

detailed cell uptake studies with these and other peptides in order to improve cell delivery of ODNs.

Aknowledgements

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