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Efficient enantiomeric analysis of primary amines and amino alcohols by high-performance liquid chromatography with precolumn derivatization using novel chiral SH-reagent *N*-(*R*)-mandelyl-(*S*)-cysteine

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Abstract

Novel *N*-acylated-(*S*)-cysteine derivative—*N*-(*R*)-mandelyl-(*S*)-cysteine (*R*-NMC), containing additional chiral center, aromatic and polar α-substituents in contrast to the traditionally used enantiomerically pure thiols, has been demonstrated to be an efficient SH-reagent for enantiomeric HPLC analysis of primary nonfunctionalized amines and amino alcohols after precolumn derivatization with *o*-phthalaldehyde. The *R*-NMC-derived isoindoles as well as adducts formed using traditional SH-reagents had a characteristic absorption maximum at 340 nm with a molar absorbance 6000 M^{-1} cm^{-1}, were stable during the HPLC-analysis and highly fluorescent allowing to detect 1 fmol of amino compound. Using diastereomeric *R*-NMC all tested amino alcohols were resolved effectively as well as nonfunctionalized amines, some of which were not resolved by a direct method on a chiral phase. Applying traditional enantiomeric *N*-acetyl-(*S*)-cysteine (NAC) only some isoindoles formed by aliphatic amino alcohols have been separated satisfactorily. The enhanced selectivity for *R*-NMC-derived isomers has been achieved, obviously, due to the involvement of the substituents at an extra chiral center into additional intramolecular interactions.

Keywords: Chiral analysis; *o*-Phthalaldehyde; Chiral thiol; Amines; Amino alcohols

1. Introduction

HPLC is an extensively used technique for quantitative analysis of diastereomers. Derivatization of α-amino acids by *o*-phthalaldehyde (OPA) and a chiral thiol leading to the formation of diastereomeric isoindoles has been widely exploited for determination of α-amino acid enantiomers. For the first time this approach was applied by using NAC and *tert*-butyloxycarbonyl-(*S*)-cysteine [1,2], however, subsequently several other chiral mercaptanes have been employed for derivatization with OPA, such as enantiomerically pure *N*-acylated (*S*)-cysteines [2,7,9,12–14], *N*-acetyl-(*R*)-penicillamine [7], (*R*)-3-mercapto-2-methylpropionic acid [5], and thiocarbohydrates [8,15]. It was found that the structure of a chiral SH-compound used for derivatization can significantly influence the resolution [2,5,7–9,16] as well as the stability of the isoindoles [5,17]. Particular attention was paid to the mechanism of chiral discrimination of diastereomeric isoindoles and the impact of intramolecular interactions such as coulomb attraction [18], hydrogen bonds [5,7,16] and π-interactions [10] has been considered. Derivatization with OPA has been successfully applied mainly for an enantiomeric analysis of α-amino acids [4,5,19,20], their amides and esters [3–5], α-alkyl-α-amino acids [3–6] and nucleoamino acids [11]. However, chiral analysis of other primary amino compounds, for example, chiral amino alcohols and especially of nonfunctionalized amines using this methodology has received just limited consideration in the literature [5,7–9,10]. Attempts to use NAC for this purpose have demonstrated low diastereoselectivity [7,9] (see also Table 1), in contrast to high resolution observed in a case of amino acids.

Recently method of enantioselective biocatalytic synthesis has been developed for preparation of several novel *N*-acyl-(*S*)-cysteines, containing additional chiral center and different α-substituents compared to the traditionally used enantiomerically

Abbreviations: k'_1 and k'_2, retention factors; α, selectivity; R_S, resolution factor; OPA, *o*-phthalaldehyde; NAC, *N*-acetyl-(*S*)-cysteine; *R*-NMC, *N*-(*R*)-mandelyl-(*S*)-cysteine

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Table 1
Comparison of NAC and *R*-NMC as chiral SH-reagents for enantiomeric analysis of amino compounds

No.[a]	NAC				*R*-NMC			
	k'_1	α	R	EC (%)[b]	k'_1	α	R	EC (%)[b]
1	26	1.00	0	30	13	1.04	1.35	37
2	16	1.00	0	27	9.4	1.05	1.35	37
3	31	1.00	0	27	20	1.05	1.42	35
4	15	1.00	0	25	21	1.02	1.1	33
5	46	1.00	0	27	23	1.03	0.90	37
6	49	1.02	0.45	27	19	1.03	1.00	33
7	2.7	1.23	3.47	30	1.8	1.10	2.24	37
8	17	1.04	0.86	25	15	1.13	4.2	27
9	13	1.03	0.81	25	10	1.13	3.5	27
10	19	1.05	1.34	22	3.4	1.06	1.81	37
11	3.0	1.11	1.61	25	7.9	1.16	3.26	27
12	3.8	1.13	2.40	20	1.5	1.08	1.63	27
13	2.3	1.13	2.16	25	2.2	1.27	4.37	30
14	26	1.06	1.76	27	10	1.05	1.53	33

[a] Structures are presented on Scheme 2.
[b] EC content of acetonitrile (v/v) in a mobile phase.

pure thiols [9]. These compounds seem to be quite promising SH-reagents for enantiomeric HPLC analysis of primary amino compounds by precolumn modification with OPA. In this paper we have studied the possibility to perform chiral HPLC analysis of primary nonfunctionalized amines and amino alcohols after their precolumn derivatization by OPA and a novel diastereomeric SH-reagent, *R*-NMC.

2. Experimental

2.1. Materials

OPA (99%) was obtained from Koch Light (UK); NAC (99%) from Sigma (USA); (*R*)-, (*S*)-phenylglycinol (98%), from Aldrich Chemie (Germany); (*R*)-, (*S*)- and (±)-1-phenylethylamine (98%), (*R*)-, (*S*)-phenylalaninol (99%), (*R*)-, (*S*)-leucinol (98%), (*R*)-, (*S*)-1-(2-naphthyl)ethylamine (99%), (*R*)-, (*S*)-2-amino-1-propanol (99%), (±)-2-amino-4-phenylbutane (98%), (±)-2-amino-6-methylheptane (98%), (±)-6-amino-2-methyl-2-heptanol hydrochloride (99%) from Fluka (Switzerland); PMSF, methanol, SDS from Merck (Germany); (±)-1-(4-chlorophenyl)ethylamine (90%) from Acros (Belgium); HPLC grade acetonitrile from Kriochrom (Russia); (±)-1-benzyl-2,2,2-trifluoroethylamine was kindly provided by Dr. V.A. Soloshonok (Institute of Oil Chemistry and Bioorganic Chemistry, Kiev, Ukraine). Preparation of penicillin acylase (PA) from *Escherichia coli* was obtained and characterized as described in [21].

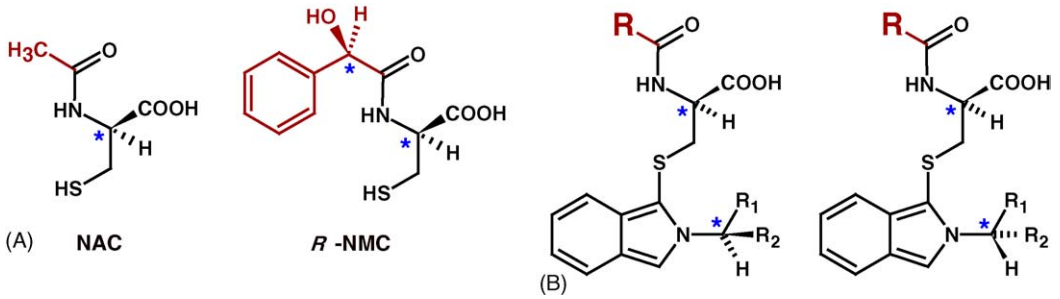
2.1.1. Synthesis of N-(R)-mandelyl-(S)-cysteine

Synthesis of *R*-NMC was performed by PA-catalyzed acyl transfer from (*R*)-mandelamide to the amino group of cysteine. The acylation was carried out in a thermostatted cell of 719 S Titrino pH-stat (Metrohm, Switzerland) at pH 9.5, 25 °C in an aqueous medium (total volume 20 ml) with 0.2 M (*S*)-Cys, 20 μM *Escherichia* PA repetitively adding acyl donor (7 × 1 mmol) every 5–10 min under permanent stirring and pH control using 2 N KOH solution. After 45 min the biocatalytic conversion was stopped by adding 5 N KOH in order to adjust pH to 12 and inactivate enzyme. Obtained mixture was filtered, the filtrate was acidified by 4 N HCl and the target product was precipitated by zinc sulfate at pH 6. Thiol was extracted by ethylacetate (3 × 5 ml) at pH 2, and organic layer was evaporated under vacuum. *R*-NMC was crystallized from an aqueous ethanol. Yield 60%, 0.61 g; e.e. > 99.9%; ^{1}H NMR (250 MHz, DMSO): δ 2.35 (t, 1H, SH), 2.85 (m, 2H, CH_2S), 4.45 (q, 1H, CH), 5.00 (s, 1H, CHCO), 6.30 (s, 1H, OH), 7.22–7.48 (m, 5H, Ph), 8.15 (d, 1H, NH), 13.0 (br. s, 1H, COOH); MS *m*/*z*: 256 (5, M + H), 238, 192, 160, 148, 132, 121, 107, 79, 77, 51.

The optical purity of *R*-NMC (e.e. > 99.9%) was demonstrated by HPLC analysis using its stereoisomer *N*-(*S*)-mandelyl-(*S*)-cysteine as a reference (*S*-NMC). *S*-NMC was synthesized in the same way as *R*-NMC using (*S*)-mandelamide as acyl donor (data not presented). Two diastereomeric thiols were analyzed on a reversed phase Phenomenex Luna 5u C-18 column (250 mm × 4.6 mm, 5 μm) with 7 mM phosphate pH 3.0, containing acetonitrile (40%, v/v) as a mobile phase, and spectrophotometric detection at 210 nm. Operational conditions: 25 °C; flow rate 0.8 ml/min. Retention time (in min): *R*-MNC (6.0), *S*-NMC (7.1).

2.2. Instrumentation

HPLC system consisted of a Waters M6000 pump, a reversed phase Phenomenex Luna 5u C-18 column (250 mm × 4.6 mm, 5 μm), a Spark Holland Marathon autosampling injector and a Perkin-Elmer LC-235 Diode Array Detector (detection at 340 nm) or Shimadzu RF-10 Axl fluorescence detector (excitation at 340 nm, emission at 450 nm). The chromatograms acquisition and processing was performed by Ampersand MultiCrom software package (Russia). The column was operated at 25 °C. Flow rate of a mobile phase was 0.8 ml/min. The mobile phase was prepared titrating a corresponding 0.8 g/l potassium dihydrogenphosphate solution in aqueous acetonitrile (contain-



Scheme 1. (A) Structure of chiral thiols: traditionally used enantiomeric *N*-acetyl-(*S*)-cysteine (NAC) and novel diastereomeric *N*-(*R*)-mandelyl-(*S*)-cysteine (*R*-NMC). (B) Structure of the stereoisomeric isoindoles formed after derivatization of primary amines with OPA and a chiral thiol.

ing acetonitrile 18–45%, v/v) with potassium hydroxide to pH 7. The formation of isoindole adducts was monitored continuously at 340 nm with a Shimadzu UV-1601 spectrophotometer at 25 °C, pH 9.6 in 0.2 M borate buffer.

The comparative enantiomeric HPLC analysis of aromatic amines by a direct method was performed using the same HPLC system and a Crownpak CR(+) column (Daicel Chemical Industries) with H_2O–$HClO_4$ (pH 2.0) as an eluent operated at 25 °C, flow rate 0.8 ml/min, with UV detection at 210 nm.

2.3. Precolumn modification

Derivatization of primary amino groups was performed in a following way: 20 μl of an amine solution (10 mM) was diluted by 940 μl of a 0.2 M borate buffer (pH 9.6), then 20 μl of a thiol (50 mM NAC or R-NMC) and 20 μl of OPA (20 mM) solution in methanol were added, resulting mixture was stirred for 15 min at room temperature, the mixture was diluted by the eluent (pH 7) and subjected to HPLC analysis. The methanol solutions of OPA and thiols were stored for a month in a dark vessel at +5 °C.

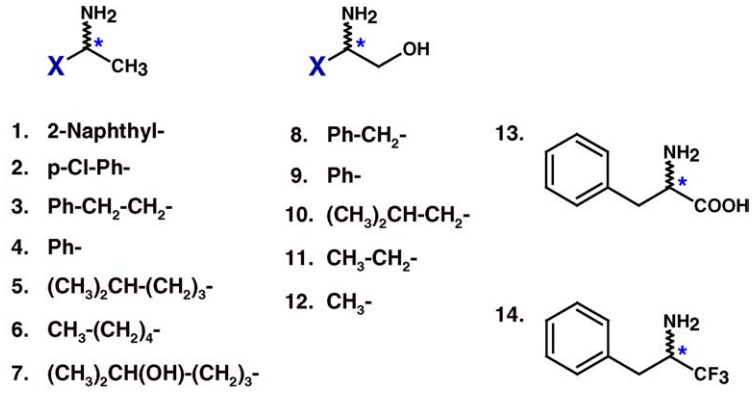
2.4. Isoindole stability

The isoindole stability was investigated at the conditions of HPLC analysis. The typical experiment was as follows: after derivatization of primary amino compound by OPA and SH-reagent a solution of the formed isoindole was diluted ten times by an eluent which has been used in HPLC analysis as a mobile phase. The obtained solution was incubated at 25 °C, aliquots of the reaction mixture were taken in a course of incubation, diluted by the eluent (pH 7) and subjected to HPLC analysis.

3. Results and discussion

A novel chiral thiol—*N*-(*R*)-mandelyl-(*S*)-cysteine, containing additional chiral center, aromatic and polar α-substituents in contrast to the traditionally used enantiomerically pure SH-reagents, and NAC (Scheme 1A) have been applied for enantiomeric analysis of aliphatic and aromatic primary amino compounds (Scheme 2). Derivatization of their amino group with OPA and a chiral thiol results in two highly fluorescent stereoisomeric isoindoles (Scheme 1B) with a characteristic absorption maximum at 340 nm. Isocratic HPLC analysis of the resulted adducts was performed on a reversed phase C-18 column (250 mm × 4 mm, 5 μm) with a spectrophotometric and fluorescence detector. Evidently the chromatographic behaviour of the formed isoindoles is determined by their structure and ionic state at the analysis conditions. Preliminary experiments have revealed decreased retention factor k' as well as improved selectivity α and resolution factor R_S for both NAC and *R*-NMC-derived isoindoles when pH of the mobile phase was shifted from an acidic (pH 3) to a neutral value (data not shown), what is consistent with the literature [16], and can be conditioned by the dissociation of the cysteine's carboxylic group. Therefore, a buffer with pH 7 has been used further as a mobile phase.

The obtained results (Table 1) demonstrate that resolution of the formed diastereomers strongly depends on both the nature of the amino component and the structure of the



Scheme 2. Structures of the investigated amino compounds.

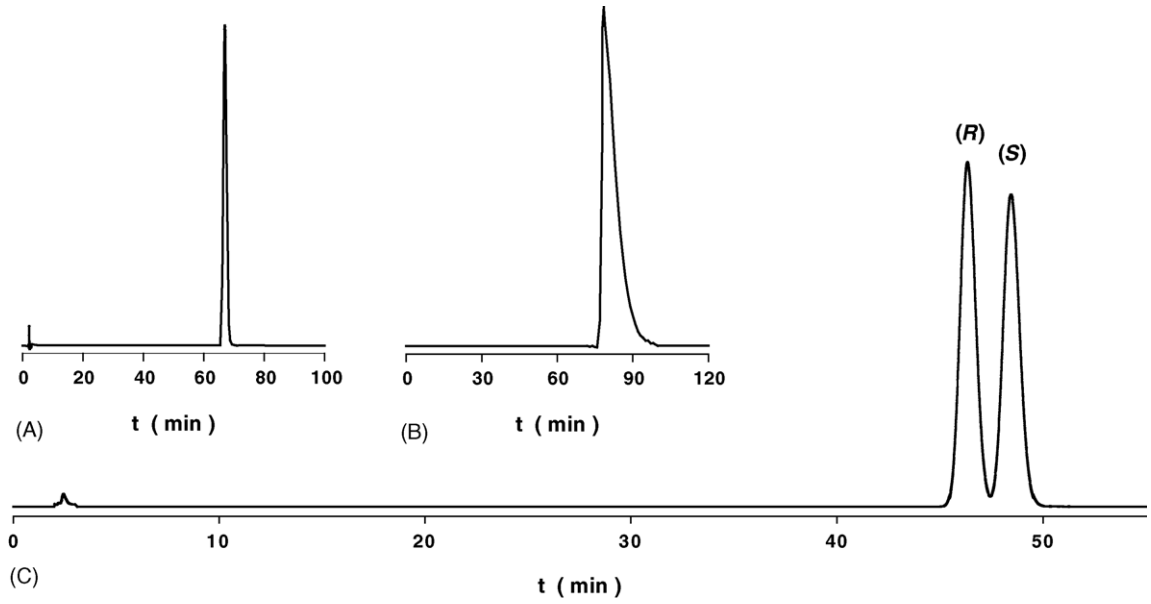


Fig. 1. Chromatograms of enantiomeric HPLC analyses: no resolution of **3** (Scheme 2 using the indirect method after its precolumn derivatization with OPA and NAC (A), no resolution of **3** using a direct analysis on Crownpak CR(+) chiral column (B), effective resolution of **3** after its derivatization with OPA and *R*-NMC (C) on a conventional reversed-phase C-18.

SH-reagent. The selectivity was excellent for amino acid **13**, and it was higher for amino alcohols than for nonfunctionalized amines. On the other hand, resolution was more efficient when functionalized diastereomeric *R*-NMC was applied instead of enantiomeric NAC. The resolution factor R_S for the NAC-derived isoindoles was good or nearly sufficient for enantiomeric analysis of some amino alcohols, whereas none of the diasteomeric adducts of nonfunctionalized amines has been separated. Fortunately, application of *R*-NMC as a chiral SH-reagent significantly enhanced selectivity: enantiomers of all amino alcohols have been resolved effectively, and acceptable or good enantioseparation has been obtained for nonfunctionalized amines, even for those which could not be resolved on a chiral stationary phase. A representative example is presented on Fig. 1.

The chiral discrimination of stereoisomeric isoindoles is based on their different conformational rigidity determined by intramolecular interactions. An elevated selectivity for amino acid- and amino alcohol-derived isoindoles compared to that for adducts formed by nonfunctionalized amines can be explained by the formation of an intramolecular hydrogen bond between the cysteine's carboxyl group and the carboxyl or hydroxyl group of these amino compounds [16]. The enhanced selectivity for all *R*-NMC-derivatives compared to NAC-adducts might be conditioned by the involvement of the chiral mandelyl residue with its aromatic moiety and OH-group into the additional intramolecular interactions. An assumption concerning the role of the additional intramolecular interactions is maintained also by the fact that a similar nature of the side chain radical of an amino compound and a thiol reagent favours resolution of the diastereomers. So, the selectivity of the NAC-derived isoindoles is more pronounced for aliphatic amino alcohols (**7**, **10**–**12**) compared to aromatic ones (**8** and **9**). On the contrary, using *R*-NMC aryl amines (**1**–**4**) and aromatic amino alcohols (**8** and **9**) are resolved more effectively compared to aliphatic ones—(**5** and **6**) and (**7**, **10**–**12**), correspondingly.

The elution order of the diastereomers formed has been determined independently using derivatized individual enantiomers. As a rule the NAC-adducts formed by β-amino alcohols were eluted in an order first *S*, then *R*, what was typical also for the hydrophobic α-amino acids [2,7]. For *R*-NMC-OPA derivatives mainly the same elution order, (*S*) before (*R*), was observed with β-amino alcohols, but in the case of nonfunctionalized amines the order was the opposite: (*R*) prior to (*S*) (presence of OH-group changes the Cahn–Ingold–Prelog nomenclature). With ε-amino alcohol **7** the (*R*), (*S*) order was observed with both thiol reagents.

The modification of all studied compounds proceeded within 10–15 min, except for **14** reacting 3 h apparently due to the low nucleophilicity of its amino group. The stability of the formed isoindoles depended on the structure of both SH-reagent and amino compound, however, all studied stereoisomeric isoindoles were totally stable at chromatographic conditions (Table 2), what is imperative for reliable HPLC analysis. Despite the fact that some stereoisomers, in principle, could display different stability (for example NAC-isoindoles with **3**, **5**, and *R*-NMC-isoindoles with **4**, **5** in Table 2), the peaks of both diastereomers formed after modification of a racemate were equal at both spectrophotometric and fluorescence detection. During derivatization procedure and HPLC analysis no racemization and kinetic resolution took place. The kinetics of isoindole formation with pure *R*- and *S*-enantiomer of an amino compound was identical at the modification conditions, namely the initial rates of isoindole formation, their maximum accumulation, and the time of isoindole's maximum accumulation coincided for *R*- and *S*-enantiomers of amino compounds at both the spectrophotometric and fluorimetric detection. The detection limits were 2–4 pmol for spectrophotometric and 0.6–2 fmol for fluorescence detection

Table 2
Stability of isoindoles at chromatographic conditions[a]

Time (h)	**1**[b]			**2**			**3**				**4**				**5**			
	NAC	*R*-NMC		NAC	*R*-NMC		NAC		*R*-NMC		NAC		*R*-NMC		NAC		*R*-NMC	
	(±)	*R*	*S*	(±)	*R*	*S*	*R*	*S*	*S*	*R*	*S*	*R*	*R*	*S*	*R*	*S*	*S*	*R*
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1	101	99	99	99	99	99	98	100	99	99	99	99	99	99	100	98	99	99
3	99	101	101	98	97	97	97	99	95	95	97	98	95	92	99	96	99	99
6	98	100	100	98	94	94	96	98	90	90	95	95	90	84	98	93	97	98
12	97	99	99	96	90	89	93	98	81	82	91	90	80	70	96	87	95	96

[a] Based on a peak area.
[b] Structures are presented on Scheme 2; the enantiomers are presented in their elution order.

Table 3
Detection limits (fmol) for NAC and NMC-derived isoindoles at a signal-to-noise ratio of 3:1

No.[a]	NAC		*R*-NMC	
	UV detection	Fluorescence detection	UV detection	Fluorescence detection
1	4000	220 (±)	2000	610 (*R*)
			2500	740 (*S*)
3	2800	5.2 (±)	5600	1.7 (*R*)
			5700	1.9 (*S*)
8	1800	1.8 (*R*)	1400	1.5 (*S*)
	2100	1.9 (*S*)	1600	1.7 (*R*)
11	380	0.48 (*S*)	530	0.81 (*R*)
	390	0.50 (*R*)	660	0.95 (*S*)
13	260	0.48 (*R*)	270	0.58 (*S*)
	300	0.60 (*S*)	280	0.60 (*R*)

[a] Structures are presented on Scheme 2; the enantiomers are presented in their elution order.

at a signal-to-noise ratio of 3:1 (Table 3), except for naphthylamine **1**, which formed weakly fluorescing isoindoles with both SH-reagents. The NAC and *R*-NMC-derived isoindoles had the same molar absorbance $\varepsilon_{340} = 6000 \pm 200\,M^{-1}\,cm^{-1}$. The linearity using these thiols was estimated over the range 50 pmol–10 nmol and 50 fmol–10 pmol of amino group with a correlation coefficient over 0.99 for spectrophotometric and fluorescence detection, correspondingly.

It should be noted, that besides amine **3** (Fig. 1) another hydrophobic amine **1** cannot be subjected to analysis on a Crownpack CR(+) chiral column due to the very high elution time (more than 4 h). Taking into account the sensibility and availability of the indirect chiral HPLC analysis based on OPA derivatization with chiral thiols, this could be considered as an eloquent example when the indirect approach has principal advantages compared to the direct method.

4. Conclusions

Use of a novel chiral SH-reagent *R*-NMC has expanded application of the enantiomeric HPLC analysis of primary amino compounds based on their precolumn derivatization by OPA. Derivatization employing this functionalized diastereomeric thiol made possible efficient chiral analysis of amino alcohols and nonfunctionalized amines, which could not be analyzed using enantiomeric NAC, and, in some cases, a direct method on a chiral column. Improved procedure is characterized by effective resolution, high sensitivity and reproducibility. By introducing additional chiral center into the thiol's structure it became possible to enlarge the resolving potential without affecting the stability and spectral properties of the formed isoindoles.

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