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Novel, Potent and Selective Chimeric FXa Inhibitors Featuring Hydrophobic P_1-Ketoamide Moieties†

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Dedicated to the Memory of Joseph E. Semple

Abstract—Judicious combination of P-region sequences of highly potent anticoagulant proteins including NAP5, NAP6, Ecotin, and Antistasin with SAR from small molecule FXa inhibitors led to a series of chimeric inhibitors of formula **1a–j**. We report herein the design, synthesis, and biological activity of this novel family of FXa inhibitors that express both high in vitro potency and superb selectivity against related serine proteases.

Factor Xa (FXa) is a trypsin-like serine protease that catalyzes the penultimate step of the coagulation cascade following initiation. Assembly of FXa with cofactor FVa on the surface of an anionic phospholipid membrane in the presence of calcium forms the prothrombinase (PTase) complex, which in turn proteolytically activates the zymogen prothrombin to thrombin (FIIa). Thrombin is the terminal serine protease of the cascade, converting soluble fibrinogen to insoluble fibrin and activating platelets that ultimately form a blood clot. Consequently, FXa, PTase, and FIIa play key roles in the regulation of normal hemostasis and abnormal intravascular thrombus development (thrombosis).[1] The development of efficacious small molecule inhibitors of thrombosis would fulfill a major unmet medical need, and continues to be an area of intensive investigation in the pharmaceutical industry.[2] Selective FXa and PTase inhibitors may have distinct therapeutic advantages over thrombin inhibitors (i.e., less potential for bleeding complications) and may ultimately provide greater safety and efficacy for both venous and arterial antithrombotic indications.

A large variety of novel, potent, and selective small molecule thrombin[3] and FXa[4] inhibitor scaffolds have emerged from our laboratories. Concurrently, the discovery of a new family of anticoagulant proteins containing the potent and selective FXa and PTase inhibitors AcAP5 (NAP5) and AcAP6 (NAP6)[5] that incorporate nontraditional hydrophobic amino acids in their corresponding P_1 recognition site led us to investigate new hybrid classes of synthetic inhibitors. In this letter, we describe the design, synthesis, and biological activity of potent chimeric FXa inhibitors **1a–j**, which feature novel P_3-*d*-arginine-P_1-hydrophobic ketoamide pharmacophores (Fig. 1).

Inhibitor Design Strategy

Certain small proteins secreted by hematophagous organisms, including NAP6 (AcAP6, Corvas, K_i FXa 996 pM, PTase 207 pM; P_3–P_1 putative reactive site sequence: R-S-F)[5] and Ecotin (UCSF, from *Escherichia coli*, K_i FXa 54 pM; P_3–P_1 sequence: S–T–M)[1b,6] demonstrate powerful anticoagulant properties in part due to their potent inhibition of FXa and prothrombinase. In contrast to related protein FXa inhibitors, including NAP5 (AcAP5, Corvas, K_i FXa 43 pM, PTase 144 pM),[5] Antistasin (Merck, ATS; K_i FXa 500 pM, PTase 114 pM),[7] and small molecule inhibitors of FXa that feature the P_3-R-P_1-R bis-cationic motif,[1,2,4a] NAP6 and ecotin utilize aromatic Phe and hydrophobic Met residues at their P_1-positions, respectively. Intrigued by the presence of such rare, nonbasic residues and cognizant of the structure–activity relationships (SAR) of synthetic FXa inhibitors,[2,4] we designed novel chimeric targets **1a–j** that feature non-basic, aromatic or hydrophobic P_1-α-ketoamide units (Fig. 1).

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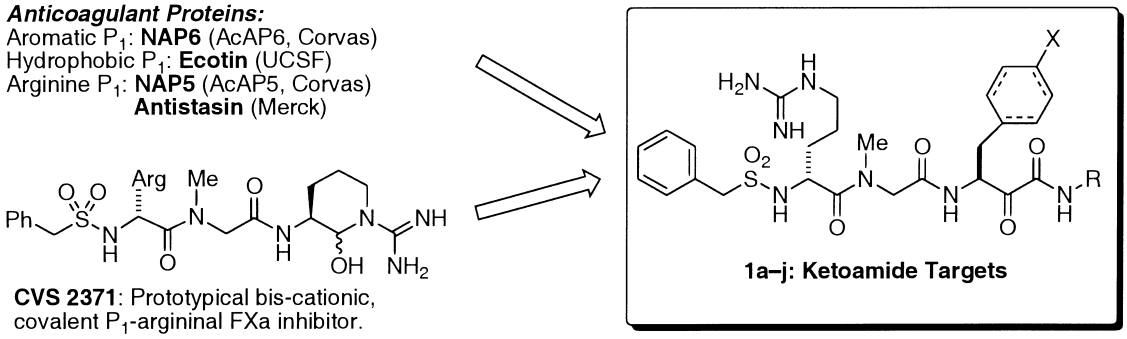


Figure 1. Design of P_1-hydrophobic ketoamide FXa inhibitors **1a**–**j**. X = H, OH, OR; dotted lines represent either aromatic or cyclohexyl rings. R denotes suitable P_1'–P_n' moieties.

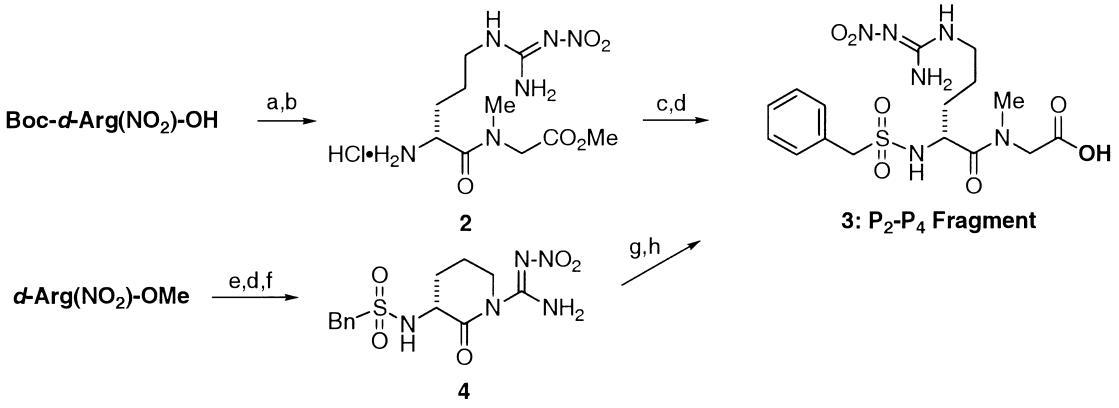
Our new targets utilize a ketoamide moiety as the transition state analogue (TSA) functionality and feature a P_3-*d*-arginine–P_1-cyclohexyl/aromatic scaffold. Based on previous SAR trends, the optimal P_4-benzyl sulfonamide and P_2-sarcosine residues were maintained.[4,8] Replacement of the highly basic P_1-arginine side chain (guanidinium cation pK_a ~12.5) with hydrophobic/aromatic residues was deemed an attractive strategy for potentially improving the PK profiles in this series of inhibitors.[4b] Furthermore, the aromatic sidechains may participate in edge-to-face and cation–π interactions at the S_1–S_1' pockets of FXa,[1,2,9] potentially conferring enhanced binding affinity and improved potency. We report herein the synthesis and biological activity of this novel family of FXa inhibitors that express high in vitro potency and superb selectivity against related serine proteases.

Chemistry

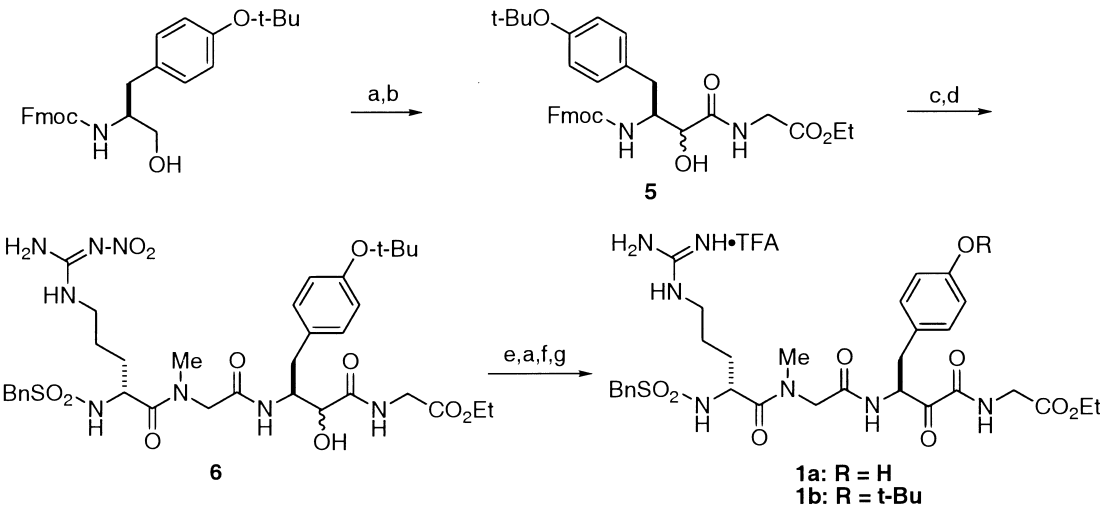
Two complementary routes to the P_2–P_4 fragment **3** are outlined in Scheme 1.[10] In the initial, somewhat capricious peptide-coupling route, commercial Boc-*d*-Arg(NO_2)–OH was elaborated over two steps to **2**, which in turn was converted to the key intermediate **3**.[8a] The yields shown were oftentimes difficult to reproduce especially on multigram scales, which led us to investigate a more reliable alternative route to **3**. A novel ring-opening strategy proceeded from *d*-Arg(NO_2)–OMe, which was converted in three steps to the acylguanidine derivative **4** in 88% overall yield. Ring-opening of **4** with the appropriate sarcosine–aluminum amide reagent followed by ester cleavage to **3** was very efficient and proved serviceable for multigram preparations of this key intermediate. To the best of our knowledge, such ring-opening processes are unprecedented.

Our approach to the P_1-Tyr ketoamides **1a**,**b** proceeded as outlined in Scheme 2. Our recently described TFA-mediated Passerini strategy[11,12] was employed for the concise construction of all key P_1–P_1' precursors. Oxidation of protected tyrosinol to the corresponding aldehyde[13] and immediate three-component reaction with ethyl isocyanoacetate and TFA in the presence of pyridine buffer directly provided multigram quantities of adduct **5** in good overall yield as a ca. 1:1 mixture of diastereomers at the newly created α-hydroxy center. Chiral HPLC analysis (Chiracel AD; isopropanol, hexane gradient) showed only two peaks, indicating retention of configuration at the original chiral center.[11a] Deprotection and coupling with acid fragment **3** delivered advanced intermediate **6**, which was processed by standard chemistry to deliver the P_1-tyrosine ketoamides **1a**,**b** in satisfactory overall yields. P_1-Phenylalanine target **1c**, a direct analogue of **1a**, was produced in a similar fashion.

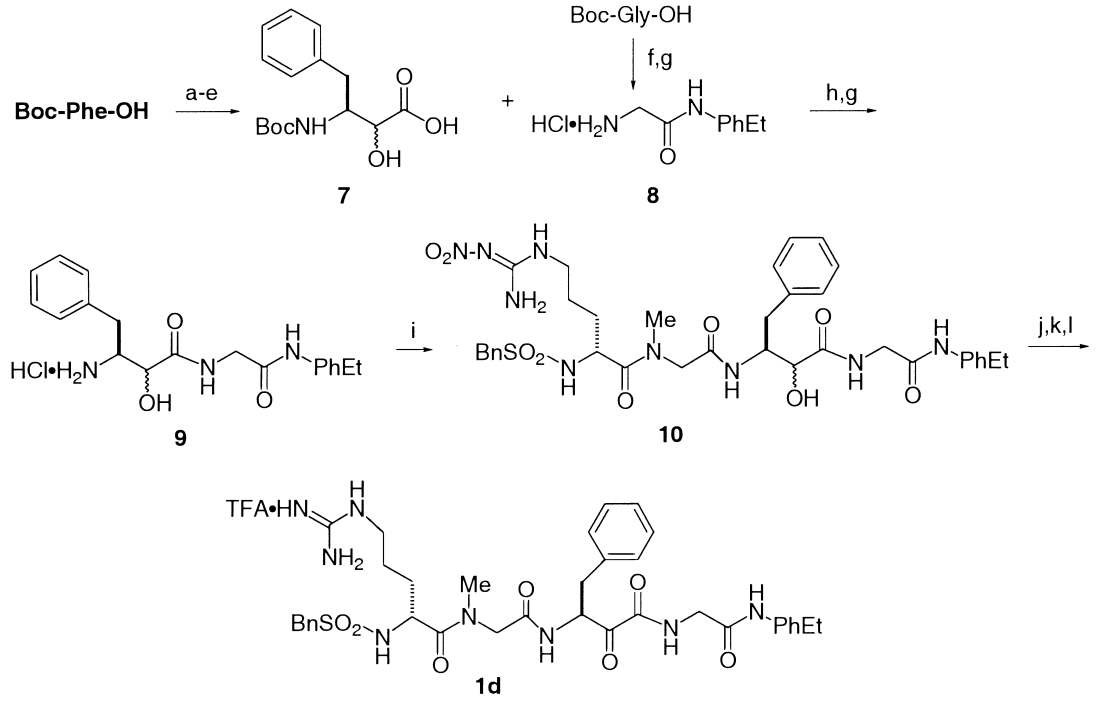
A convergent approach to the extended P_1-Phe ketoamide **1d** is outlined in Scheme 3. Boc-Phe–OH was elaborated



Scheme 1. Reagents and conditions: (a) HCl·Sar–OMe, EDC, HOBt, NMM, CH_3CN, 0 °C to rt; (b) HCl, MeOH, 0 °C to rt, 52–72% overall; (c) $BnSO_2Cl$, Et_3N, CH_3CN, 0 °C to rt, 45–72%; (d) LiOH, MeOH, H_2O, 0 °C to rt, Dowex H^+, ~quant; (e) $BnSO_2Cl$, NMM, DMF, 88%; (f) EDC, HOBt, NMM, MeCN, ~quant.; (g) Me_3Al, HCl·Sar-OtBu, THF, 0 °C to rt, 75%-quant; (h) TFA, CH_2Cl_2, 0 °C to rt, ~quant.



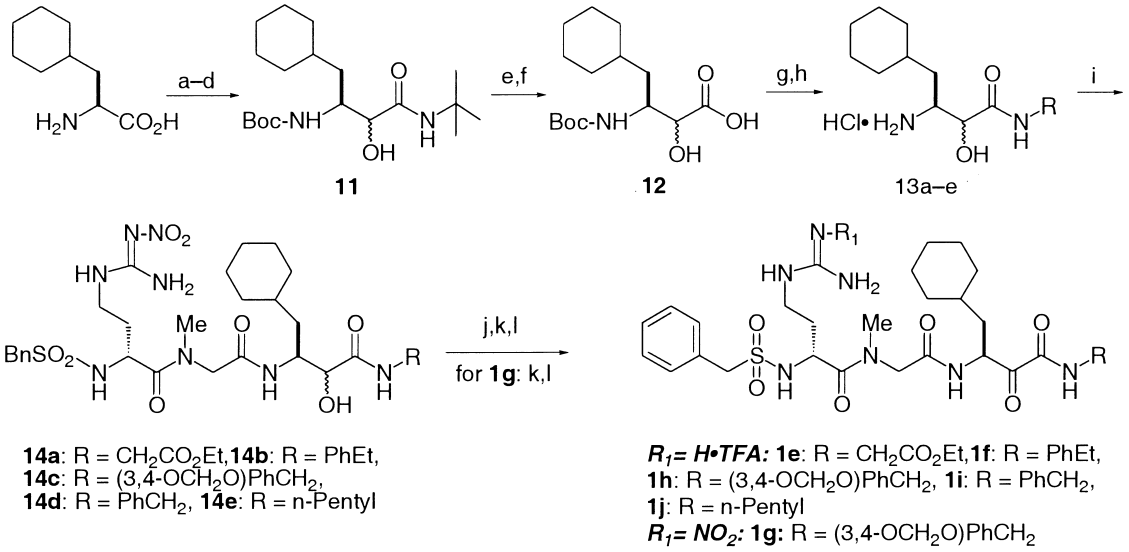
Scheme 2. Reagents and conditions: (a) EDC, Cl_2CHCO_2H, DMSO, toluene, ~5 °C to rt, ~quant; (b) $CNCH_2CO_2Et$, TFA, pyridine, CH_2Cl_2, 0 °C to rt, 69%; (c) Et_2NH, CH_2Cl_2, 0 °C to rt, ~quant; (d) **3**, DIPC, HOBt, CH_3CN, rt, 49%; (e) H_2, Pd/C, EtOH, HOAc, H_2O, ~quant; (f) RP-HPLC purification, 42%; (g) for R = H (**1a**): TFA, CH_2Cl_2, 0 °C to rt, ~quant.



Scheme 3. Reagents and conditions: (a) MeNH(OMe)·HCl, HOBt, EDC, DIEA, MeCN, quant; (b) $LiAlH_4$, THF, −78 °C; H^+, 99%; (c) *tert*-BuNC, TFA, 2,4,6-collidine, CH_2Cl_2, 0 °C to rt, 78%, (Passerini rxn. on ~70 g scale); (d) 6 N HCl, reflux; (e) Boc_2O, Na_2CO_3, dioxane, H_2O; H^+, 93% for two steps; (f) $PhEtNH_2$, EDC, HOBt, NMM, MeCN, 0 °C to rt, quant; (g) HCl, EtOAc, 0 °C to rt, ~quant.; (h) EDC, HOBt, NMM, MeCN, 0 °C to rt, 95%; (i) **3**, HATU, HOAt, NMM, MeCN, 0 °C to rt, ~quant; (j) H_2, Pd/C, EtOH, HOAc, H_2O, ~quant; (k) EDC, Cl_2CHCO_2H, DMSO, toluene, ~5 °C to rt; (l) RP-HPLC, 34–43% overall.

in five steps to the norstatine derivative **7** on a ca. 20–30 g scale, again featuring a key Passerini reaction step. P_1'–P_2' fragment **8** was assembled and coupled with **7** to afford **9** which, in turn, was coupled with **3** to produce the advanced intermediate **10**. Elaboration of **10** over three steps provided the P_4–P_2' target **1d**. This convergent (12-linear steps longest sequence) methodology proved reliable and was scalable, affording multigram quantities of the final target **1d** for further biological evaluations.

The synthesis of the P_1-cyclohexylalanine ketoamides **1e–j** is outlined in Scheme 4. Cyclohexylalanine was converted in four steps to **11** which after hydrolysis and protection afforded the Chx-norstatine derivative **12**. Condensation of **12** with various amines and deprotection gave the P_1–P_1' fragments **13a–e**. Standard coupling of acid **3** with amines **13a–e** gave the corresponding products **14a–e**, which were converted as outlined to targets **1e–j**.



Scheme 4. Reagents and conditions: (a) Boc_2O, 1 N NaOH, MeCN, 16 h, quant; (b) MeNH(OMe)·HCl, HOBt, EDC, DIEA, MeCN, quant; (c) $LiAlH_4$, THF, −78 °C; H+, 93%; (d) *tert*-BuNC, TFA, pyridine, 0 °C to rt, 46%; (e) 6 N HCl, reflux ; (f) Boc_2O, K_2CO_3, dioxane, H_2O; H^+, 72% for two steps; (g) Couple P_1'-amine, EDC, HOBt, DMF, 75–90%; (h) 4 N HCl, dioxane, 0 °C, ~quant; (i) **3**, HATU or EDC, HOBt, DIEA, DMF, 62–80%; (j) H_2, Pd/C, EtOH, H_2O, AcOH, ~quant; (k) EDC, DMSO, DCA, toluene, 0 °C to rt; (l) RP-HPLC, 45–63%.

Biological Activity

The in vitro activity of the P_1-ketoamides **1a–j** along with the argininal standard **CVS 2371**[4b,8] is summarized in Table 1. **CVS 2371** demonstrated high in vitro potency on both amidolytic FXa and PTase,[4c] but lacked selectivity on plasmin and trypsin. Good to excellent FXa inhibition was observed for the new targets, with IC_{50}'s = 0.78–46 nM. Within a defined P_4–P_2' scaffold, variations at P_1 produced the following SAR results: ChxAla (**1e**, 2.1 nM) > Phe (**1c**, 6.6 nM) ≥ Tyr (**1a**, 7.6 nM) >> Tyr(*t*-Bu) (**1b**, 246 nM). Amongst the four different P_1 groups prepared, the hydrophobic P_1-ChxAla target **1e** expressed optimal activity. Further exploration of **1e** at the P_1'–P_2' region led to superior inhibitors, with **1h** (IC_{50} = 0.78 nM) emerging as the top candidate. Very high selectivity against thrombin, plasmin and trypsin was noted for most targets. Leading candidates from series **1** showed classical slow binding kinetics of inhibition, the reported IC_{50} values are at T = 30 min. Limited available data indicates these new targets are only modestly active as PTase inhibitors, with IC_{50}'s = 9–29 nM.

The inhibitory potency of ketoamides **1a–j** may result from numerous energetically favorable interactions throughout the active site P_4–P_2' manifold, including canonical β-sheet, hydrophobic, van der Waals and aromatic edge-to-face interactions.[4,9] Binding to FXa probably occurs in a normal extended substrate-like mode, with the P_3-*d*-Arg residue participating in key cation–π interactions at the S_4 pocket.[1b,2c,4] Docking of our new P_1 residues at either the S_1 (most likely) or S_1' specificity pockets is tentative, but the biological results suggest that such unconventional aromatic/hydrophobic binding interactions are viable in the FXa active site.

Table 1. In vitro activity of P_1-hydrophobic ketoamide FXa inhibitors **1a–j**

Compound	P_1-Sidechain	R	IC_{50} Values (nM)[a]				
			FXa	PTase	Plasmin	FIIa	Hu Trypsin
CVS 2371 Reference			0.86	3.4	1200	>2500	74.7
1a	4-(OH)$PhCH_2$	CH_2CO_2Et	7.63	29.2	Inact.	>2500	Inact.
1b	4-(*t*-BuO)$PhCH_2$	CH_2CO_2Et	246	ND	>2500	Inact.	Inact.
1c	$PhCH_2$	CH_2CO_2Et	6.6	25.1	Inact.	>2500	>2500
1d	$PhCH_2$	$CH_2CONHPhEt$	7.89	25.9	Inact.	>2500	Inact.
1e	*c*-$C_6H_{11}CH_2$	CH_2CO_2Et	2.13	ND	Inact.	>2500	Inact.
1f	*c*-$C_6H_{11}CH_2$	PhEt	13.2	ND	Inact.	2210	>2500
1g	c-$C_6H_{11}CH_2$	(3,4-OCH_2O)$PhCH_2$	46.4[b]	ND	Inact.	>2500	Inact.
1h	*c*-$C_6H_{11}CH_2$	(3,4-OCH_2O)$PhCH_2$	0.78	9.0	>2500	1720	>2500
1i	*c*-$C_6H_{11}CH_2$	$PhCH_2$	3.26	ND	>2500	1960	2500
1j	*c*-$C_6H_{11}CH_2$	n-Pentyl	1.87	ND	>2500	3830	163

[a]Concentration of compounds **1a–j** necessary to inhibit FXa, PTase, plasmin, FIIa, and human trypsin cleavage of the chromogenic substrates described in ref 3 by 50%. Reported value for each compound is from a single IC_{50} determination which confirmed initial range values.
[b]**1g** is the P_3-*d*-Arg(NO_2) derivative (see Scheme 4).

Conclusion

Judicious combination of structural information gleaned from naturally occurring small protein anticoagulants[5–7] with evolving SAR trends from small molecule antithrombotic inhibitors[2,4] led us to design and synthesize a series of novel covalent FXa inhibitors **1a–j**, which incorporate hydrophobic or aromatic P_1-ketoamide moieties. Application of our recently described Passerini technology[11] expedited the assembly of these targets and facilitated rapid SAR development. In vitro evaluation revealed potent inhibitors of FXa, which demonstrated good to excellent selectivity profiles towards thrombin (FIIa), plasmin and trypsin. Derivative **1h** (IC_{50} FXa = 0.78 nM, PTase = 9.0 nM) emerged as the top candidate and constitutes a novel structural paradigm. Numerous active site interactions coupled with optimal P_1-substitution and presentation geometry are important for conferring good FXa inhibitory potency and high selectivity into this class.

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