CONFIRMATION OF *IN VITRO* SYNTHESIS OF 2-DEOXY-*SCYLLO*-INOSOSE, THE EARLIEST INTERMEDIATE IN THE BIOSYNTHESIS OF 2-DEOXYSTREPTAMINE, USING CELL FREE PREPARATIONS OF *STREPTOMYCES FRADIAE*†

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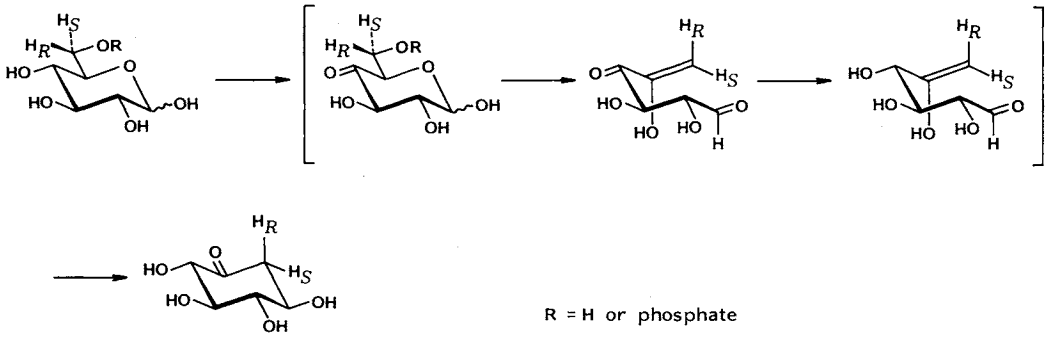
(Received for publication December 2, 1991)

The *in vitro* activity of formation of 2-deoxy-*scyllo*-inosose, the earliest intermediate of the 2-deoxystreptamine biosynthesis, was confirmed in the precipitate fraction obtained by 30~45% saturation of ammonium sulfate from the 15,000 × *g* supernatant of the sonicated *Streptomyces fradiae* IFO 13147 cells.

2-Deoxystreptamine, the characteristic cyclitol, is a central part of clinically important glycoside-containing antibiotics.[1,2] It is found only in these antibiotics and is a typical secondary metabolite. It was well-established that 2-deoxystreptamine is biosynthesized from D-glucose *via* the C-C bond formation between C-1 and C-6 as shown in Fig. 1.[3,4] The stereochemistry of this ring closure was also determined.[5,6] The first intermediate produced by this C-C bond formation reaction was proposed as a non-aminogenous cyclitol, 2-deoxy-*scyllo*-inosose, based on the supplementation experiments with idiotrophic mutants of antibiotic-producing microorganism.[7] The formation of 2-deoxy-*scyllo*-inosose by non-producing blocked mutants was suggested by experiments with an ingenious technique of cosynthesis.[8] As described in our recent paper,[9] we have directly and physically detected the existence of 2-deoxy-*scyllo*-inosose in the cultured broths of several antibiotics-producing microorganisms by the GC-MS SIM methodology.

From the mechanistic point of view, we postulated that the crucial cyclization reaction in the biosynthesis of 2-deoxystreptamine is similar to the dehydroquinate synthase reaction in the shikimate

Fig. 1. The postulated mechanism of 2-deoxy-*scyllo*-inosose formation in the 2-deoxystreptamine biosynthesis.



† Biochemical studies on 2-deoxy-*scyllo*-inosose, an early intermediate in the biosynthesis of 2-deoxystreptamine III.

pathway.[5,6,10] In the biosynthesis of 2-deoxystreptamine, the phosphate group of D-glucose-6-phosphate is believed to play an important role in the formation of an enol-type intermediate by β-elimination.[5,6] For such β-elimination, a prior oxidation of the C-4 hydroxyl group to a ketone was shown to be involved.[11,12] Thus, D-glucose-6-phosphate is apparently the most plausible precursor to the cyclization product, 2-deoxy-*scyllo*-inosose. Until now, however, neither the real precursor nor an oxido-reduction cofactor has been unambiguously identified. To get more precise insight into the reaction mechanism, an *in vitro* system for the 2-deoxy-*scyllo*-inosose formation seems to be necessary. So far, the enzyme(s) involved in the 2-deoxy-*scyllo*-inosose formation has neither been isolated, nor has a cell-free system mediating this conversion been established. The transamination reaction which converts 2-deoxy-*scyllo*-inosose to 2-deoxy-*scyllo*-inosamine was shown to involve a pyridoxal-phosphate dependent transamination enzyme.[13,14]

This paper describes the first confirmation of the synthesis of 2-deoxy-*scyllo*-insose using a cell-free preparation derived from a neomycin-producing strain, *Streptomyces fradiae* IFO 13147.

Materials and Methods

Chemicals

D-Glucose and D-glucose-6-phosphate di-potassium salt were obtained from commercial sources. D-[6,6-$^2H_2$]Glucose was prepared according to the LEMIEUX's method.[15] D-[6,6-$^2H_2$]Glucose-6-phosphate was prepared using a method adopted from the chemical synthesis of D-glucose-6-phosphate using D-[6,6-$^2H_2$]glucose as a starting material.[16] NAD and NADP were purchased from Sigma or Oriental Yeast (Tokyo). All other chemicals were of commercial origin and were used without further purification. Distilled, deionized water was used for preparation of buffer solutions. 2-Deoxy-*syclo*-inosose was prepared by a previously reported method.[17] Glucose oxidase was obtained from Nagase Biochemicals (Tokyo).

Instrumentations

The GC-MS selected ion monitoring spectra were obtained as described previously using a Shimadzu LKB 9020DF mass spectrometer.[9] HPLC was performed on a Hitachi L-6000 pump connected with a Hitachi L-4000 UV detector. HPLC data were processed and recorded on a Hitachi D-2500 integrator.

Quantitative Analyses of 2-Deoxy-*scyllo*-inosose

(1) By the GC-MS SIM Method: The amount of 2-deoxy-*scyllo*-inosose in the enzyme reaction was quantitated by the GC-MS SIM analysis as described previously.[9]

(2) By the HPLC Method: A standard curve for quantitative analysis was prepared with 0.5 ml buffer solution (buffer A: 50 mM Tris-HCl, pH 7.5) of various concentration of 2-deoxy-*scyllo*-inosose. To each solution was added 2 mg of *O*-(4-nitrobenzyl)hydroxylamine hydrochloride in 0.2 ml of pyridine and the whole mixture was heated to 60°C for 2 hours with occasional shaking. After cooling to room temperature, the mixture was flushed with N_2 gas to remove the solvent. The residue was dissolved with 250 μl of MeOH. The mixture was subjected to thin-layer chromatography (Merck Kieselgel F_{254}) developed with $CHCl_3$-MeOH (5:1). The band containing 2-deoxy-*scyllo*-inosose- and 2-deoxyglucose *O*-(4-nitrobenzyl)oxime derivatives (Rf 0.25 to 0.4) was scraped off and the compounds were eluted from the silica gel with $CHCl_3$-MeOH (5:1). The eluate was evaporated to dryness and the concentrate was dissolved in 50 μl of MeOH. A portion (5 μl) of the solution was analyzed on the aforementioned HPLC apparatus equipped with a TSKgel ODS-80T_M CTR column (4.6 mm i.d. × 10 cm). The eluate was monitored by UV absorbance at 262 nm. The mobile phase consisted of 18% of MeOH in deionized water.

Microorganism and Culture

The organism *S. fradiae* IFO 13147 strain was maintained on agar slants of BENNETT medium

consisting of glucose 10 g; yeast extract (Oxoid) 1 g; Ehrlich meat extract (Kyokuto Yakuhin, Tokyo) 1 g; NZ-amine type A (Humco Sheffield Chemical) 2 g; and agar 20 g per liter (pH 7.3). A saline suspension of whole cells and spores from the slant (8 or 9 day's) was added to 100 ml of ISP No. 2 liquid medium consisting of glucose 4 g; yeast extract (Oxoid) 4 g; malt extract (Oxoid) 10 g per liter (pH 7.3), in a 500-ml Erlenmeyer flask. Cultures were grown on a rotary shaker at 28 to 29°C.

The Cell-free Preparation

The 88-hour cultures of a neomycin-producing strain *S. fradiae* IFO 13147 were harvested by centrifugation (10,000 × *g*, 10 minutes), and the cells were washed twice with buffer A (at 4°C). The cells were suspended in buffer B (buffer B: 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM of Co^{2+} and Mg^{2+}) in a ratio of 0.4 g of wet cells per 1 ml of buffer, and sonicated for 20 minutes at 0°C (iced water bath). The sonicate was centrifuged at 15,000 × *g* for 30 minutes and the resulting supernatant was used as the cell-free preparation (28 mg/ml protein). Protein concentration was determined by the LOWRY's procedure.[18]

Ammonium Sulfate Precipitation

The cell-free preparation was fractionated by $(NH_4)_2SO_4$ precipitation in a standard manner. The precipitates were recovered by centrifugation (10,000 × *g*, 30 minutes) and redissolved in a minimum amount of buffer B, and then dialyzed against the same buffer for 24 hours at 4°C to afford the active fraction (15 mg/ml).

Incubation Method

A portion (1.0 ml) of the enzyme preparation was mixed with 0.1 ml of 100 mM solution of a substrate candidate (D-glucose, D-glucose-6-phosphate di-potassium salt, D-[6,6-$^2H_2$]glucose, or D-[6,6-$^2H_2$]glucose-6-phosphate di-potassium salt), and 0.1 ml of either 50 mM (for GC-MS SIM analysis) solution or 25 mM (for HPLC analysis) solution of NAD or NADP. Incubation was carried out at 37°C with shaking for 16 hours. Aliquots (0.5 ml) were dispensed for quantitative analysis of 2-deoxy-*scyllo*-inosose.

For the GC-MS SIM analysis, each assay mixture was lyophilized. Pyridine (100 μl), hexamethyldisilazane (50 μl) and chlorotrimethylsilane (50 μl) were added and the resulting mixture was heated to 50°C for several minutes. After leaving at room temperature for 1 hour, 100 μl of hexane was added to the mixture followed by centrifugation to remove the salts formed. The resulting supernatant was subjected to the GC-MS analysis with monitoring *m/z* 452, 362 and 450, 360.[9] For the control experiment, a portion of the cell-free preparation was heated in a boiling water bath for 5 minutes. The heat-treated preparation was incubated as described above and the reaction was similarly analyzed.

For HPLC analysis, 40 μl of glucose oxidase solution in buffer B (0.8 mg) was added to each sample followed by incubation at 37°C for 1 hour. The reaction was terminated by addition of MeOH (0.2 ml) followed by shaking for several minutes. The precipitates (denaturated proteins) were removed by centrifugation (2,500 × *g*, 10 minutes). To the supernatant thus obtained was added 5 μl of a solution (200 μg/ml) of 2-deoxy-D-glucose as an internal standard. *O*-(4-Nitrobenzyl)hydroxylamine hydrochloride (2 mg) in 0.2 ml of pyridine was added to form a spectrophotometrically visible derivative. The whole mixture was heated to 60°C for 2 hours with periodic shaking. After cooling to room temperature, the solvent was removed by flushing N_2 gas. The residue was dissolved to 250 μl of MeOH and filtered through a cellulose acetate filter (Advantec TOYO dismic-13CP020, 0.20 μm hole). The filtrate was analyzed by TLC and HPLC as described above. The amount of 2-deoxy-*scyllo*-inosose *O*-(4-nitrobenzyl)oxime was quantitatively estimated by comparing the intensity of internal standard 2-deoxyglucose *O*-(4-nitrobenzyl)oxime derivatives.

Results and Discussion

In vitro synthesis of 2-deoxy-*scyllo*-inosose was attempted first with the cell-free preparation derived from a neomycin-producing strain *Streptomyces fradiae* IFO 13147. The 15,000 × *g* supernatant of the

Table 1. Amounts of 2-deoxy-*scyllo*-inosose found by the cell-free reaction system of *Streptomyces fradiae* IFO 13147.

Enzyme	Substrate[a]	Cofactor[b]	2-Deoxy-*scyllo*-inosose (μg/ml)					
			Run 1		Run 2		Run 3	
			m/z 452[c]	*m/z* 362[c]	*m/z* 452[c]	*m/z* 362[c]	*m/z* 452[c]	*m/z* 362[c]
Cell-free extract	Control	None	3.0	1.8	0.5	0.1	1.8	0.0
Heat treated cell-free extract	D-[6,6-$^2H_2$]Glucose	None	0.7	0.0	0.3	0.0	—	—
Heat treated cell-free extract	D-[6,6-$^2H_2$]Glucose-6-phosphate	None	0.6	0.0	0.1	0.0	—	—
Cell-free extract	D-[6,6-$^2H_2$]Glucose	None	6.2	(19.4)[d]	3.9	2.5	5.5	4.3
Cell-free extract	D-[6,6-$^2H_2$]Glucose-6-phosphate	None	6.2	7.0	6.5	4.4	7.2	3.4
Cell-free extract	D-[6,6-$^2H_2$]Glucose	NAD	3.6	(12.3)[d]	0.3	0.0	0.3	0.6
Cell-free extract	D-[6,6-$^2H_2$]Glucose-6-phosphate	NAD	3.1	(12.4)[d]	1.7	2.7	2.6	1.5
Cell-free extract	D-[6,6-$^2H_2$]Glucose	NADP	—	—	—	—	0.6	0.2
Cell-free extract	D-[6,6-$^2H_2$]Glucose-6-phosphate	NADP	—	—	—	—	4.2	4.0

[a] Substrate concentration 10 mM.
[b] Cofactor concentration 5 mM.
[c] SIM analysis was independently carried out at *m/z* 452 and 362.
[d] The amounts in the parenthesis may be due to the coexistence of other contaminants.

cell sonicate was used as an enzyme preparation. Incubations were carried out in the metal-supplemented buffer (buffer B) as described in the Materials and Methods section in the presence of either D-[6,6-$^2H_2$]glucose or D-[6,6-$^2H_2$]glucose-6-phosphate. The amount of 2-deoxy-*scyllo*-inosose in each reaction mixture was analyzed with the GC-MS SIM technique by monitoring the ions at *m/z* 452 and 362. Further, a portion of the cell-free preparation was heated in a boiling water bath for 5 minutes and the residual conversion activity of the resulting heat-treated extract was analyzed in the same manner. The amount of 2-deoxy-*scyllo*-inosose found in the control experiments corresponds to the endogenous 2-deoxy-*scyllo*-inosose in the cell-free preparation (Table 1). With the incubations of the cell-free preparation in the presence of plausible substrates (D-[6,6-$^2H_2$]glucose or D-[6,6-$^2H_2$]glucose-6-phosphate), a significant increase of 2-deoxy-*scyllo*-inosose was observed. That the amounts estimated by the ion of *m/z* 352 were less than those estimated by the ion of *m/z* 452 was not surprising, because two fragmentation pathways are available from the molecular ion *m/z* 452 giving rise to *m/z* 352 and *m/z* 351 as well.[9] Essentially no 2-deoxy-*scyllo*-inosose was detected in the samples derived from the incubation with the heat-treated preparation. Thus, it appears that the cell-free preparation did produce 2-deoxy-*scyllo*-inosose from D-[6,6-$^2H_2$]glucose and D-[6,6-$^2H_2$]glucose-6-phosphate and the principle(s) responsible for this transformation was inactivated by heating. D-Glucose-6-phosphate seemed to be a better substrate than D-glucose under these conditions (runs 2 and 3). A difficulty not allowing clear differentiation might be attributed to the presence of other enzymes involved in the carbohydrate metabolism.

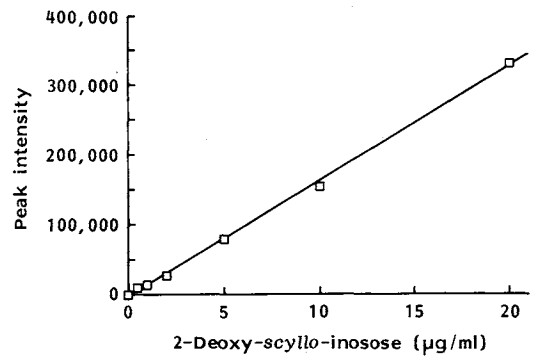
As to the cofactor involvement proposed for the oxido-reduction, NAD and NADP were examined by adding each separately to the cell-free preparation. As can be seen from the Table 1, no clear improvement of conversion was observed in either case. It may be anticipated that the formation of 2-deoxy-*scyllo*-inosose seems to require only catalytic amount of cofactor as does the dehydroquinate synthase reaction.[10] It seems, therefore, that the supplementation of the possible cofactors did not necessarily induce significant difference from the unsupplemented under these conditions. Somewhat curious was that addition of NAD to the reaction mixture turned out to reduce the formation of

2-deoxy-*scyllo*-inosose. While rational for this was not clear, supplementation of NAD to the cell-free preparation might facilitate the reactions of other NAD-dependent enzymes, *e.g.* D-glucose-6-phosphate dehydrogenase. Even though some ambiguities were involved, the formation of 2-deoxy-*scyllo*-inosose seemed apparent, especially in the cases of cofactor unsupplemented incubation with either D-glucose or D-glucose-6-phosphate.

To obtain a clearer definition of the observations, similar experiments were carried out with enzyme fractions obtained by ammonium sulfate precipitation of the cell-free preparation.

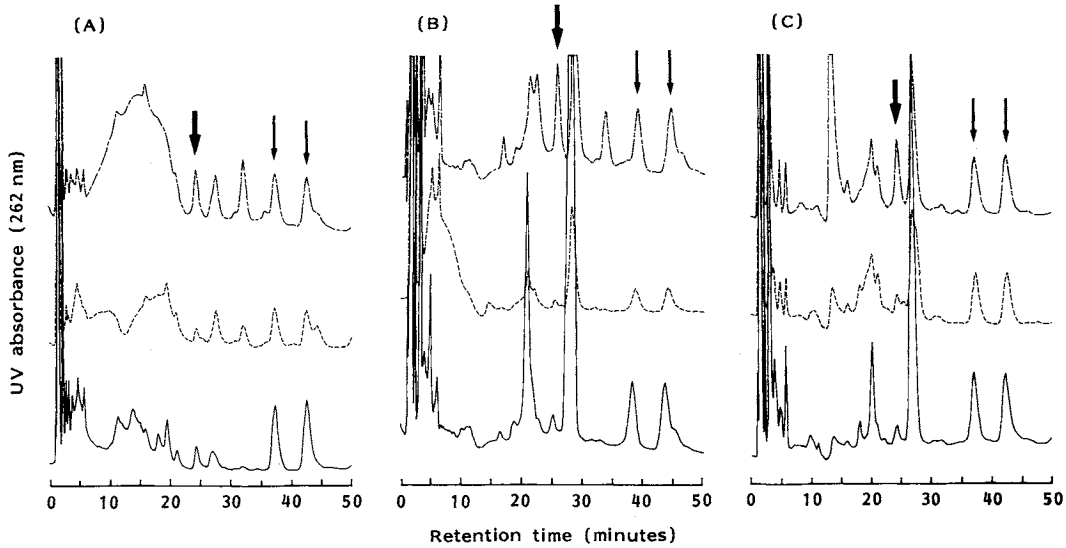
Applicability of the HPLC method for the quantitative analysis of 2-deoxy-*scyllo*-inosose was first tested and the results clearly demonstrated its reliability as displayed with the standard curve in Fig. 2. Reproducibility of this method was confirmed by the duplicated experiments. By using 2-deoxy-D-glucose as an internal standard, this HPLC method allows the quantitation of 2-deoxy-*scyllo*-inosose with a similar sensitivity (up to 0.2 μg/ml) to the GC-MS SIM method.[9]

Fig. 2. Quantitative analysis of 2-deoxy-*scyllo*-inosose *O*-(4-nitrobenzyl)oxime derivative.



This method was used to monitor the reactions of the ammonium sulfate precipitation with various substrates in the presence and absence of a plausible cofactor. Since it was anticipated that the presence of D-glucose (but not D-glucose-6-phosphate) might interfere with the clear differentiation of the

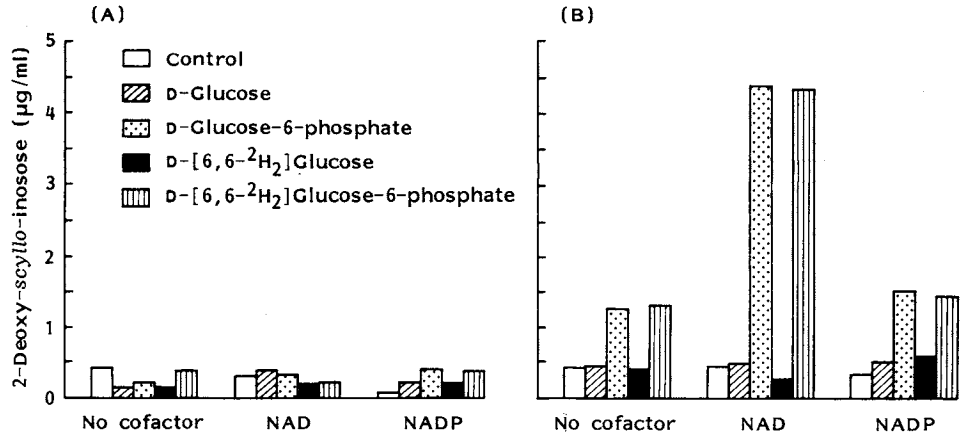
Fig. 3. HPLC profiles of the *in vitro* reactions using an enzyme fraction (30~45% $(NH_4)_2SO_4$ saturation) for 2-deoxy-*scyllo*-inosose synthesis under various conditions.



The peak of 2-deoxy-*scyllo*-inosose *O*-(4-nitrobenzyl)oxime is indicated by a large arrow. The peaks of 2-deoxy-D-glucose *O*-(4-nitrobenzyl)oxime are indicated by small arrows. Condition A: no cofactor added. Condition B: NAD added. Condition C: NADP added. Solid line: no substrate added. Dotted line: D-glucose added. Dashed line: D-glucose-6-phosphate added.

Fig. 4. Formation of 2-deoxy-*scyllo*-inosose.

(A) With an enzyme fraction precipitated by 30% $(NH_4)_2SO_4$ saturation. (B) With an enzyme fraction precipitated by 30~45% $(NH_4)_2SO_4$ saturation.



2-deoxy-*scyllo*-inosose oxime in the HPLC traces by forming D-glucose oxime, the residual D-glucose in the assay mixtures was oxidized with D-glucose oxidase. The resulting D-gluconate was supposed to be inactive to the oxime formation. As can been seen in Fig. 4(A) and 4(B), 2-deoxy-*scyllo*-inosose was in fact formed significantly by the enzyme fraction of 30~45% ammonium sulfate saturation from D-glucose-6-phosphate but not from D-glucose. No conversion activity was observed in the 45~100% saturation fraction. The ammonium sulfate fractionation procedure appears to have eliminated some enzymes which might otherwise interfere clear observation of 2-deoxy-*scyllo*-inosose formation. Thus, D-glucose-6-phosphate was determined to be the real substrate for the 2-deoxy-*scyllo*-inosose synthesizing enzyme. This result is quite reasonable, since, as discussed earlier, the formation of 2-deoxy-*scyllo*-inosose was anticipated to be similar to the dehydroquinate synthase reaction and the phosphate group is known to serve as a good leaving group in various biological transformations.

NAD was a much better cofactor than NADP. Again, the precipitation procedure seemed effective in the removal of some NAD dependent enzymes. This is interesting because this observation may further support a similarity of the 2-deoxy-*scyllo*-inosose-synthesizing enzyme, which may be called as 2-deoxy-*scyllo*-inosose synthase as we proposed previously,[5,6] to the dehydroquinate synthase. NADP was shown to facilitate slightly the formation of 2-deoxy-*scyllo*-inosose under these conditions, but the reason for this is not clear at present. A purified enzyme system is awaited for more detailed analysis.

In conclusion, the present experiments clearly demonstrated for the first time the *in vitro* transformation of D-glucose-6-phosphate (rather than D-glucose) in the presence of NAD (rather than NADP) into 2-deoxy-*scyllo*-inosose with the cell-free preparation of *S. fradiae* IFO 13147. The approach described here may allow more detailed studies of these biochemically important transformations on the molecular as well as atomic levels. The low conversion rate may be due to rather low activity of the present strain for the antibiotic production. Therefore, investigations using other strains may well be quite intriguing. Further purification of the 2-deoxy-*scyllo*-inosose synthase enzyme is currently underway.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education,

Science and Culture, and by a Research Grant from the Japan Antibiotics Research Association.

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