


METHODS FOR EXTRACTION AND ISOLATION OF AGATHISFLAVONE FROM *Poincianella pyramidalis*Bruna O. do Nascimento^a and Jorge M. David^{a,*} ^aInstituto de Química, Universidade Federal da Bahia, 40170-290 Salvador – BA, Brasil

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Agathisflavone is a natural biflavone with restricted occurrence in plants. This biflavone presents several biological activities and can be important to developing new drugs. Obtaining such a molecule on a large scale for *in vivo* tests is a challenge. To date, there are no reports regarding its synthesis; thus, it is important to improve and develop methods for the purification of this bioactive compound from natural sources. Previous studies indicated that the leaves of *Poincianella pyramidalis* (Leguminosae) had agathisflavone predominantly. However, obtaining this compound requires some steps of chromatographic procedures and extraction with organic solvents. This paper compares four procedures for the purification of agathisflavone from its leaves, two by traditional chromatographic techniques and by complexation with inorganic bases. The purification in the automatic flash chromatography in two steps provides a high-yield extraction; the two procedures using alkaline extraction and the traditional method reduced to three steps provided the compound with similar yields. The biflavone was identified by TLC, and HPLC analysis showed that it was isolated with > 99% of purity. Thus, the applied methodologies provided the purification of this compound employing less volume of solvents, the number of chromatographic column procedures, and time-consuming, with increased yields.

Keywords: agathisflavone; *Poincianella pyramidalis*; isolation; bioflavonoids; biflavone.

INTRODUCTION

Agathisflavone (**1**) is a biflavone firstly isolated from *Agathis palmerstonii* (Araucariaceae),¹ and it also occurs in other plant families such as Anacardiaceae, Burseraceae, Clusiaceae, Leguminosae, and Ochnaceae. This compound shows different biological activities, and it is important to the development of new drugs due to several biological and pharmacological activities, including the neuroprotective, modulating the astrocytic injury response and glial scar formation, stimulating neural reorganization.²⁻⁴ Moreover, *in vivo* and *in vitro* studies indicate the absence of toxicity for this biflavone.⁵ Besides its importance in drug discovery, obtaining this biflavonoid in substantial amounts for *in vivo* tests is challenging. To date, there are no reports regarding its synthesis, and a unique tentative of its total synthesis leads to a methyl derivative with low yields.⁶ Consequently, the only way to obtain it is from natural sources.

Poincianella pyramidalis (Leguminosae) is a Brazilian medicinal plant popularly known as “catingueira”, due to its predominance in the “caatinga” vegetation from the northeastern Brazilian region.^{7,8} This plant was previously classified as *Caesalpinia pyramidalis*, and it was segregated from others in the genus *Poincianella*. Studies dealing with the leaves of *P. pyramidalis* chemical composition indicated they contain usual natural products such as triterpene saponins, flavonoids, cinnamic derivatives, and the main compounds, the bioflavonoids.^{9,10} A LC-APCI-MS analysis of leaf extract demonstrated that only the specimens collected in Bahia state collectively showed the presence predominantly of agathisflavone (**1**) with concentrations ranging from 0.01 to 1% (dry mass), besides amentoflavone (**2**), sequoiaflavone (**3**), and podocarpusflavone A (**4**) (Figure 1).¹¹

In these studies, agathisflavone was obtained from *P. pyramidalis* after some chromatographic procedures after plant extraction with organic solvents.^{10,11} It is already known that, in general, obtaining metabolites from natural sources requires time-consuming and labor-intensive procedures in extraction and isolation steps due to the low concentration of these metabolites present in plant tissues.¹² Despite

the variety of methods used to obtain compounds from natural sources, there is still an urgent need to improve and develop high-yield, selective, and rapid methods to purify bioactive compounds from organisms. This paper compares four procedures for the purification of agathisflavone from the leaves of *P. pyramidalis*, two by traditional chromatographic techniques and two by complexation with inorganic bases.

MATERIALS AND METHODS

General procedures

Methanol and dichloromethane were furnished by TEDIA, and chloride acid and calcium hydroxide were from Vetec and Sigma-Aldrich. The isolation procedures were monitored by Silica TLC plates (Macherey–Nagel or Fluka) through 254 nm UV lamp viewing (Cabinet Spectroline). Agathisflavone was also purified by fully automated Biotage equipment of flash chromatography (mod. Isolera One System) using pre-packed flash columns and an internal wavelength (200–400nm) detector. ¹H NMR spectra were recorded at 500. MHz and ¹³C NMR spectra at 125 MHz on a Bruker equipment mod Avance III 500 (11,75T), chemical shifts were recorded in δ (ppm) from the residue solvent peak relative to TMS. The evaluation of the isolated biflavonoid purity was carried out by HPLC–DAD Shimadzu[®] equipment (mod. Nexera XR) with a scan range from 200 to 400 nm. A commercial octadecyl group (C18) column (Shim-pack PREP-ODS (H) KIT; 250.0×4.6 mm ID, 5 μ m, Shimadzu) was employed.

Plant material

The leaves of *Poincianella pyramidalis* were collected in the surroundings of Feira de Santana (12°15'55.1 “S 38°56'54.7 “W) and Valente (11°25'49.8 “S 39°29'53.1 “W), Bahia, Brazil. The voucher was deposited at Herbário Alexandre Leal da Costa of Instituto de Biologia of Universidade Federal da Bahia under number 240291. The authors obtained the authorization to access the plant through

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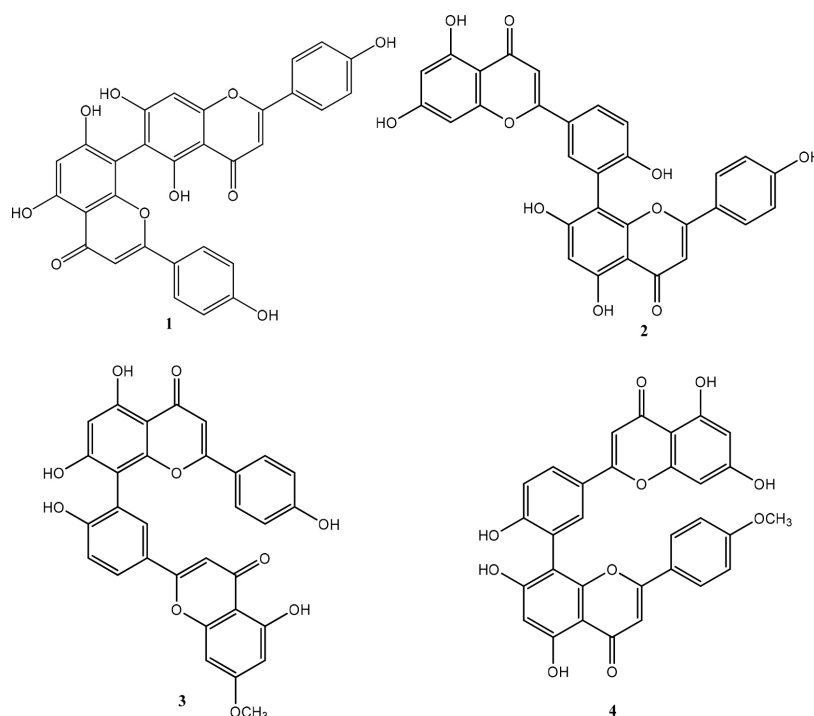


Figure 1. Structure of agastiflavone (1), amentoflavone (2), sequoiaflavone (3), and podocarpusflavone A (4), biflavones found in *P. pyramidalis* leaves

the Brazilian System for the Management of Genetic Heritage and Associated Traditional Knowledge – SISGEN (#A55D19D).

Extraction and Isolation

The leaves of *P. pyramidalis* were dried in an oven at 45 °C for 72 h and then grounded in a mixer. All the described methods used the MeOH extract obtained by maceration for 48 h at room temp of the dried and grounded leaves.

Method A: Chromatographic purifications

Isolation by partition and column chromatography A.1

This procedure is considered the traditional technique for phytochemical isolation and purification of specialized metabolites. The maceration in MeOH (2x 1 L) of 200 g leaves of plant material furnished 20.8 g of methanolic extract. The MeOH extract was submitted to partition between hexane, and the alcoholic soluble fraction was diluted with 30% of water and subsequently partitioned with CHCl_3 (759 mg). The hydromethanolic phase was concentrated in the rota evaporator to remove the MeOH excess, and, sequentially, it was extracted with EtOAc, furnishing 1.58 g of EtOAc-soluble fraction. This fraction was chromatographed in a column over Silica gel (Sigma-Aldrich, 40–63 μm particle size) and eluted with mixtures of CHCl_3 :MeOH. The fractions eluted with CHCl_3 :MeOH (9:1) furnished 428.0 mg of agathisflavone with yields of 0.21% from dried leaves and 26.4% of the EtOAc-soluble fraction the MeOH extract.

Automated Medium pressure liquid chromatography (MPLC)- A.2

From 25 g of dried and grounded leaves, 1 g of 2.5197 g of the obtained MeOH extract by maceration was subjected to automated flash chromatography using a pre-packed C-18 reverse phase of flash column cartridge of 25 g (Figure 2a). The sample diluted in MeOH (15 mL), after the adsorption to the same C-18 phase, was added to the cartridge samplet space (Figure 2b), and then the cartridge was connected to the equipment (Figure 2c). The elution with binary mixtures of H_2O :MeOH of 9:1; 6:4, 1:1, and 100% MeOH resulted

in four fractions of 300 mL each, collected on manual mode with a flow rate of 50 mL min^{-1} . The monitoring of this elution through UV-detector at 254 nm permitted the identification of the fraction rich in phenolic components; in this case, the third fraction was eluted in 1:1 (Figure 2d). The fraction eluted with MeOH: H_2O 1:1, after removing the methanol in the rota evaporator, was partitioned with EtOAc, providing 244 mg of EtOAc-soluble fraction. Its comparative TLC plate employing a standard revealed the presence of agathisflavone. Therefore, this phase was submitted to a flash silica gel CC using mixtures of CHCl_3 :MeOH in increasing polarities (seven fractions of 30 mL each) that permitted obtain 14,8 mg of agathisflavone in fraction eluted with 9:1 with yields of 1.48% related to the crude extract and 0.0592% concerning the dried plant material.

Method B: Alkaline extraction of biflavone

Direct treatment of the extract with base B.1

The methanol extract (2.5 g) obtained from 25 g leaves was solubilized in 50 mL of 10% calcium hydroxide aqueous solution, and the mixture was left overnight at room temperature, resulting in a suspension that was sequentially filtered through a funnel containing a thin layer of Celite on the paper filter. The yellow filtrate obtained was slowly acidified with concentrated hydrochloric acid until the pH reached 4–5. After a few minutes, these procedures furnished a precipitate, and the TLC indicated it is agathisflavone compared with a pure standard. The solid was then filtered and rinsed with distilled water. This technique permitted obtaining 3.5 mg of agathisflavone with 0.0136% yield related to the plant material and 0.136% related to the crude extract.

Treatment of the partitioned extract with base B.2

From 50 g of plant material, 3.5 g of crude extract was obtained by maceration with MeOH. The extract was solubilized in MeOH: H_2O (7:3) and submitted to partition with CHCl_3 , furnishing 857 mg of CHCl_3 soluble fraction. In sequence, this fraction was also submitted to the same procedure applied to the crude methanolic extract described in item B.1. This procedure furnished 6.5 mg of agathisflavone, 0.013% of

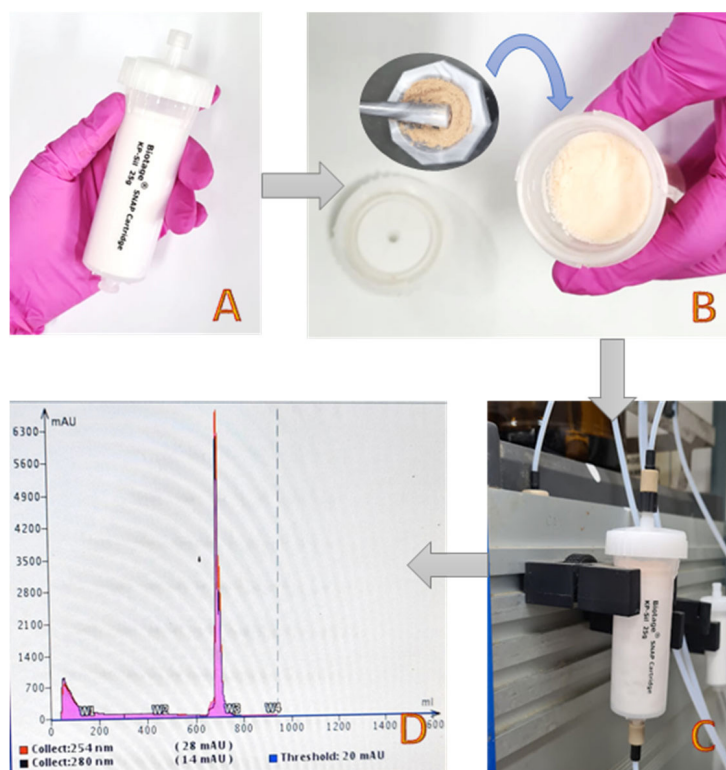


Figure 2. Scheme for the obtention of the enriched fraction of agathisflavone (method A2). A- 25 g cartridge with C-18 RP, B- addition of the dry adsorbed sample in C-18 on the sample of the cartridge, C- Connection of the cartridge to equipment, and D- The screen of the equipment with the separation monitored in 254 nm indicating the high signal in the third fraction rich in polyphenols

yield related to the plant material, 0.185% related to the crude extract, and 0.758% related to CHCl_3 soluble fraction.

Agathisflavone (1). Amorphous yellow solid, m.p 310-311 °C, ^1H NMR (500 MHz, CD_3OD): Unit I: d 6.72 (s, 1H, H-3); d 6.40 (s, 1H, H-8); d 7.94 (d, 2H, J = 8.2 Hz, H-2'/H-6'); d 6.99 (d, 2H, J = 8.2 Hz, H-3'/H-5'); Unit II: d 6.70 (s, 1H, H-3); d 6.62 (s, 1H, H-6); d 7.57 (d, 1H, J = 8.4 Hz, H-2'/H-6'); d 6.78 (d, 1H, J = 8.4 Hz, H-3'/H-5'); ^{13}C NMR (125 MHz, CD_3OD): Unit I: d 165.0 (C, C-2); d 103.0 (CH, C-3); d 183.1 (C, C-4); d 162.4 (C, C-5); d 104.0 (CH, C-6); d 162.5 (C, C-7); d 98.6 (C, C-8); d 164.6 (C, C-9); d 104.3 (C, C-10); d 121.8 (C, C-1'); d 128.2 (CH, C-2'/C-6'); d 115.8 (CH, C-3'/C-5'); d 161.1 (C, C-4'); Unit II: d 164.7 (C, C-2); d 102.5 (CH, C-3); d 182.7 (C, C-4); d 162.7 (C, C-5); d 102.5 (CH, C-6); d 162.5 (C, C-7); d 99.7 (CH, C-8); d 156.6 (C, C-9); d 104.9 (C, C-10); d 122.0 (C, C-1'); d 128.2 (CH, C-2'/C-6'); d 115.8 (C, C-3'/C-5'); d 162.0 (C, C-4').

High-Performance Liquid Chromatography (HPLC) Analysis for agathisflavone

To evaluate the purity of the obtained agathisflavone, the biflavonoid isolated by the four methods were submitted to HPLC-DAD analysis using the followed method: 10 min of running time, eluted in gradient, 0-8 min, 90-100%, 0.1% aqueous acetic acid:MeOH as mobile phase (v/v) and 2 min 100% MeOH, 0.5 mL min^{-1} of flow rate and 2 μl of each sample injection (Figure 3).

RESULTS AND DISCUSSION

Since previous studies indicated that agathisflavone was the main compound in the content of biflavones in different specimens of *P. pyramidalis*,¹¹ we decided to apply other strategies to obtain this compound with high yields and few lab steps. Previously, the

best results for isolating agathisflavone among other bioflavonoids from *P. pyramidalis* leaves were obtained after at least three chromatographic columns. After the hexane partition, the MeOH extract obtained from the leaves was partitioned with chloroform, furnishing the CHCl_3 soluble fractions submitted to silica gel CCs and a Sephadex LH-20 permeation column using a mixture of different solvents. These procedures furnished enriched phenol fractions and permitted to obtain 0.015% of agathisflavone related to the leaves.¹¹

In this study, firstly, considering the interaction of agathisflavone with the stationary phase due to the presence of six phenolic hydroxyl groups, it was expected that the employment of reverse phase column this biflavonoid could be isolated faster than using the standard method with the no-bonded stationary phase. Subsequently, without using any pre-treatment or partition, the extract was submitted to the reverse phase column. As a result, a phase with this compound impure obtained required just another step toward the final purification. However, in this procedure, few steps were employed to obtain the pure biflavonoid. A traditional chromatographic procedure with fewer steps was also developed; in this case, the EtOAc soluble fraction of the MeOH extract was employed (A.1), and the yield was lower than the preconcentration with the silica reverse phase (A.2).

The alkaline treatment to obtain flavonoids as a precipitate is known but is commonly employed for citrus peels and aboeto.¹³ However, for leaf extracts, this procedure is unusual.

The treatment with $\text{Ca}(\text{OH})_2$ removes the acidic hydrogens from the phenolic groups of flavones, and the resulted salt can be soluble in aqueous media. These two methodologies applied furnished rapid isolation of agathisflavone with a similar yield to the traditional chromatographic procedure (Table 1).

The agathisflavone obtained was identified by TLC and HPLC analysis compared to the previously isolated standard. Besides, the UV spectra obtained from the DAD detector (200-400 nm) in the HPLC analysis were also compared (Figure 4).

Table 1. Summary of the steps and yields of agathisflavone purification by chromatographic (A) and direct base extractions (B)

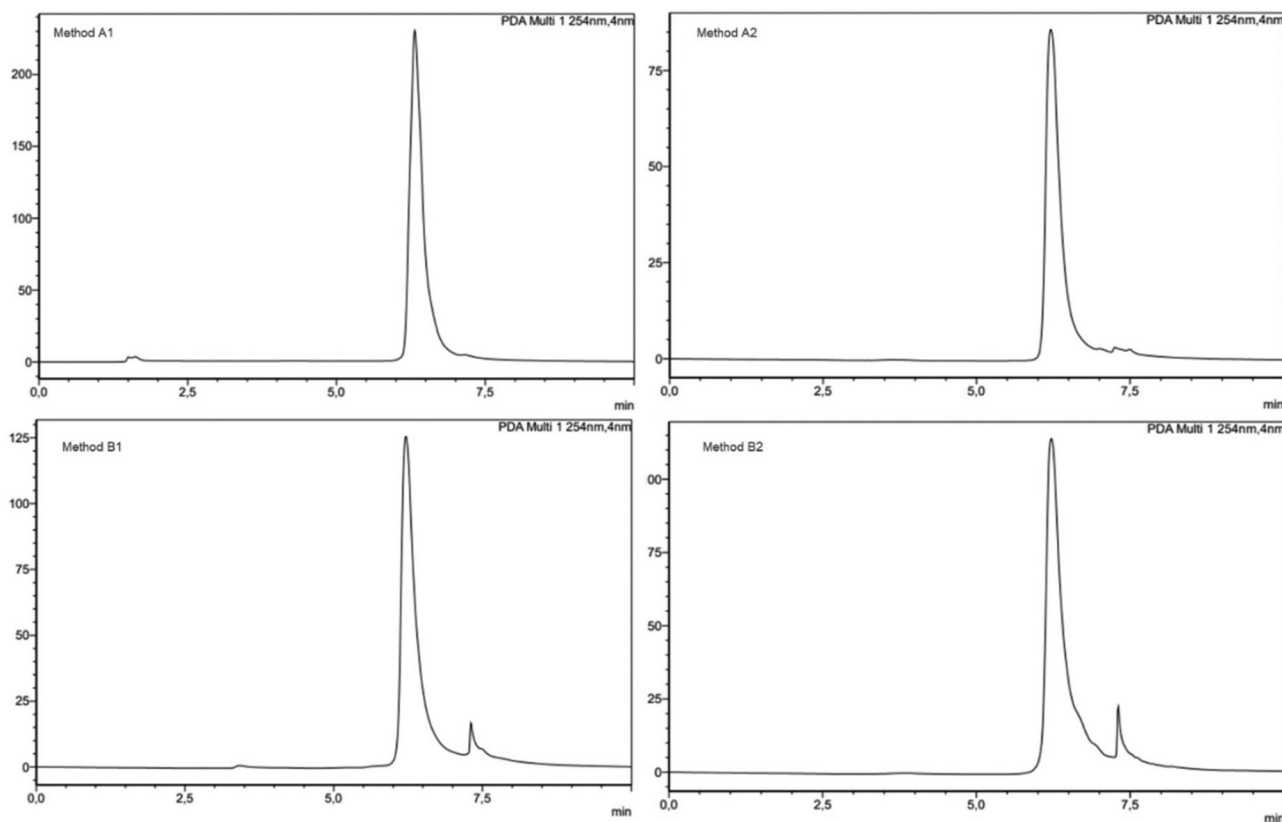
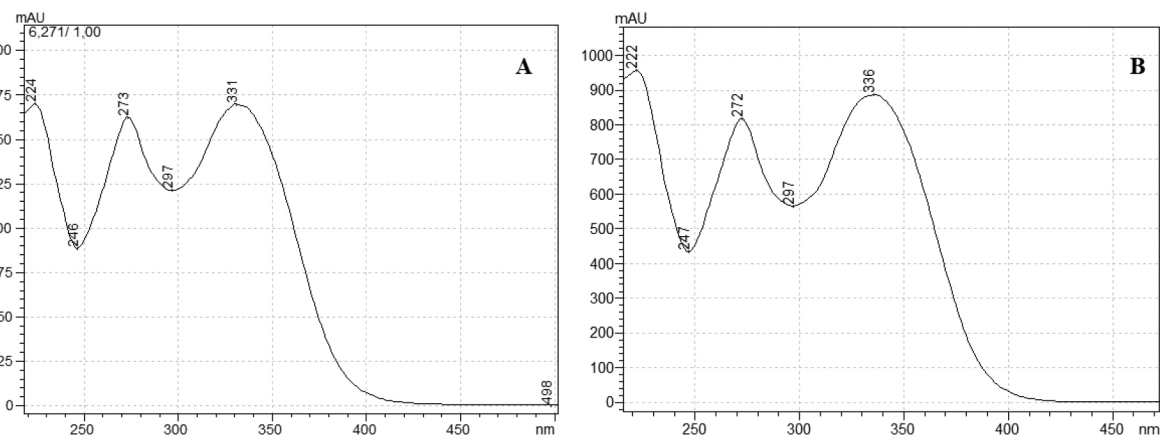
	Literature	Method A		Method B	
		1	2	1	2
Partition of crude extracts	+	++	-	-	+
Treatment with base	-	-	-	+	+
columns	+++	+	++	-	-
Total of steps	4	3	2	1	2
Yield (%)	0.015	0.014	0.060	0.014	0.014

The isolates were also identified by NMR data (mono and bidimensional techniques), and the data obtained are similar to the previously published ones. ^1H NMR is more specific for identifying organic compounds, but HPLC or GC analysis is commonly

employed for organic purity determination, especially for natural compounds. The HPLC analysis of the agathisflavone isolated using the four procedures indicates that this biflavone was obtained by the procedures A.1, B.1, and B.2 with 99.838%, 99.302%, and 99.088% of purity, respectively. Furthermore, procedure A.2 permitted obtaining it with >99.9% purity. These data were achieved relatively by the integral of the peaks displayed in the chromatographic analysis.

CONCLUSIONS

The employment of these methodologies with reduced steps showed that the purification of agathisflavone from methanolic extracts of *P. pyramidalis* leaves in the automatic RP flash chromatography provides a higher yield, just in two steps. However, this kind of equipment is not always available. Alkaline extraction with $\text{Ca}(\text{OH})_2$ is an option, considering the yield with or without

**Figure 3.** Chromatograms of agathisflavone obtained from each method applied**Figure 4.** Ultraviolet spectra of agathisflavone obtained from the isolated procedures (A) and standard (B)

partition showed around 0.014% of yields, similar to previous studies. The advantage of bases in aqueous media is the reduction of employment of more solvents and silica, which is greener. Thus, the applied methodologies showed advantages over the agathisflavone isolation less solvent employed due to the number of chromatographic column procedures, less time-consuming, and the yield of the finished product was increased.

SUPPLEMENTARY MATERIAL

The NMR spectra of this work are available at <http://quimicanova.sbq.org.br>, as a PDF file, with free access.

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