

Isolation, Characterization and Antibacterial Activity Screening of Anthocyanidine Glycosides from *Alchornea Cordifolia* (Schumach. and Thonn.) Mull. Arg. Leaves

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Abstract: From the ethanolic extract of the leaves of *Alchornea cordifolia* (Schumach. and Thonn.) Mull. Arg., 5-methyl 4'-propenoxy anthocyanidines 7-O- β -D – diglucopyranoside was isolated. The structure was elucidated using NMR spectroscopy in combination with IR and MS spectral data. Antibacterial studies showed that the isolated compound successfully inhibited *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. This result authenticates the use of the plant in phytomedicine for disease prevention and treatment of infections.

Keywords: *Alchornea cordifolia*, Anthocyanidine glucoside, Antibacterial activity, Phytomedicine.

Introduction

Anthocyanidines are widely distributed natural products exhibiting a broad spectrum of pharmacological profile. They were found to be potent antiplasmodial agents. Besides the cytotoxic, antioxidant and antiviral activities, anthocyanidines also possess antibacterial activities^{1,2}

As part of an ongoing search for biologically active secondary metabolites from the Rain Forest Biodiversity of Nigeria, *Alchornea cordifolia* (Schumach. and Thonn.) Mull. Arg. (Euphorbiaceae) was selected for studies because of its multipurpose utilization as fodder, food and medicine. *A. cordifolia* is a multi-stemmed shrub or small tree; sometimes climbing but occasionally grows as an erect spreading plant, up to 5 m high and 30 cm girth⁴. The leaves, roots and stem bark extracts are used extensively in traditional medicine in the preparation of drugs for urinary, respiratory and gastro intestinal disorders³. Slurry from the fruits is administered for asthma and cough. A decoction of the leaves is used as eye lotion⁵. The leaves and stem bark when powdered are used in the treatment of ringworms

and other skin infections⁶. There are many convergence in the traditional use of *Alchornea cordifolia* through out tropical Africa as topical anti-inflammatory; chancre, yaws, wounds, ulcers, dental caries and plaques, toothache, gum inflammation and conjunctivitis⁵⁻⁸.

It is distributed sporadically in the rain forest of Southern Nigeria and the roots and leaves are used as a carminative in Nigeria folk medicine⁵. However, very little is known about the chemical constituents of *A. cordifolia* and so far there have been few previous phytochemical investigations which reported the isolation and structural elucidation of gallic acid, ellagic acid and protocatechic acid from the plant⁹. Phytoconstituents such as steroids, phenolic compounds, flavonoids, flavones, tannins, xanthenes and alkaloids have been isolated from *A. cordifolia*¹⁰. Recently, a new flavonol glycoside isorhamnetin-3-O- β -D-xyloside was isolated from the leaves of *A. cordifolia*¹¹. As part of our chemical studies on Nigeria medicinal plants, we describe herein the isolation of a new anthocyanidine glycoside 5'-Methyl 4' Propenoxy anthocyanidines 7-O- β -D-diglucopyranoside (**1**) along with an antioxidant 5'-Methyl 4', 3, 5, 7 tetrahydroxy anthocyanidine (**2**). In addition, we investigate the antibacterial activity of the propenoxy anthocyanidine diglucopyranoside (**1**) isolated from *A. cordifolia* leaves.

Experimental

The IR spectra were determined on a Thermo Nicolet Nexus 470 FT – IR spectrometer. The ¹H NMR and ¹³C NMR spectra were recorded on a Bruker Avance 400 FT spectrometer for ¹H NMR and 75 FT spectrometer for ¹³C NMR, using TMS as internal standard. Chemical shifts are expressed in parts per million.

LC–ESIMS spectra were determined in the positive ion mode on a PE Biosystem API 165 single quadrupole instruments; HRESIMS (positive ion mode) spectra was recorded on a Thermo Finniga MAT 95 XL mass spectrometer. Column chromatography was carried out with silica gel (200-300 mesh) and to monitor the preparative separations, analytical thin layer chromatography (TLC) was performed at room temperature on pre-coated 0.25 mm thick silica gel 60 F₂₅₄ aluminum plates 20 x 20 cm Merck, Darmstadt Germany.

Plant materials

The fresh leaves of *Alchornea cordifolia* were collected from an uncultivated farmland in Umudike, Abia State, Nigeria on 6th February, 2008. The plant samples (fruits, seeds and leaves) were identified by Dr. A Nmeregini of Taxonomy Section, Forestry Department Michael Okpara University of Agriculture, Umudike, Nigeria. A voucher Specimen No: AC/552 has been deposited at the Forestry Department, Herbarium of the University.

Extraction and isolation of plant materials

Plant materials were treated and analyzed at the Chemistry Laboratory, Michael Okpara University of Agriculture, Umudike, Nigeria. The leaves (1 kg) were dried on the laboratory bench for 10 days. The dry sample was milled and ground into powder (910 g) using Thomas Wiley Machine (model 5 USA).

The powdered plant sample (500 g) was packed into a soxhlet apparatus (2 L) and extracted exhaustively with 1000 mL ethanol for 24 h. The ethanol extract was concentrated using a rotary evaporator at room temperature, and left on the laboratory bench for 2 days to obtain dark green pigment (50.3 g). The column was packed with silica gel and eluted with methanol, chloroform and petroleum ether (20:30:50) to afford a green amorphous solid (0.83 g). The green amorphous solid was recrystallized from hexane to afford compound **1**; green amorphous solid (0.36 g). Thin layer chromatography (chloroform: methanol(7: 3)

iodine vapor shows the presence of one spot. (R_f 0.62). IR ν_{\max} 34.01 cm^{-1} (OH) 2926 cm^{-1} (CH_2), 1616 cm^{-1} (C=C aromatic) and 1097 cm^{-1} (C–O ether). HREIMS m/z 647.5367 calculated for $\text{C}_{31}\text{H}_{37}\text{O}_{15}$ (m/z 648) and base peak m/z 57.0709 calculated for $\text{C}_3\text{H}_5\text{O}$. (m/z 57). ^1H NMR and ^{13}C NMR of compound **1** were presented in Table 1.

Table 1. ^1H (400 MHz) and ^{13}C NMR (75 MHz) of compound (**1**).

Position	δC		δH		
	Chemical shift δ	Carbon	Chemical shift δ	Multiplicity	Proton
1					
2	130.956	C			
3	130.320	C	4.1213	1Hbs	OH
4	130.108	C	7.2609	1Hs	C–H
5	129.800	C–OH	4.1510	1Hbs	OH
6	128.893	C	7.5155	1Hs	C–H
7	128.168	C –			
8	128.893	C	7.5380	1Hs	C–H
9	127.984	C			
10	127.984	C			
1'	128.168	C			
2'	130.320	C	7.6938	1Hs	C–H
3'	130.956	C	7.5158	1Hs	C–H
4'	128.168	C			
5'	129.800	C			
6'	130.320	C	7.6938	1Hs	C–H
1''	128.893	CH	5.3266	1Hd	C–H
2''	127.984	CH	5.3360	1Hd	C–H
3''	22.774	CH_3	0.8803	3Hd	CH_3
4''	24.957	CH_3	0.8962	3Hd	CH_3
Glucose					
1	77.418	CH	4.2187	1Hs	CH
2	77.100	CH	4.2725	1Hs	CH
3	68.987	HC–OH	4.2832	1Hm	HC–OH
4	68.256	HC–OH	4.3022	1Hm	HC–OH
5	76.783	C–H	5.3442	1Hs	C–H
6	62.191	CH_2OH	5.3492	2Hs	
1'	77.418	CH	4.2187	1Hs	C–H
2'	77.100	HC–OH	4.2725	1Hm	H–C–OH
3'	68.987	HC–OH	4.2832	1Hm	H–C–OH
4'	68.256	H–C–OH	4.3022	1Hm	H–C–OH
5'	76.783	C –	5.3360	1Hs	C–H
6'	62.191	$\text{CH}_2\text{–OH}$	5.3497	2Hs	$\text{CH}_2\text{–OH}$

Bioassay procedure

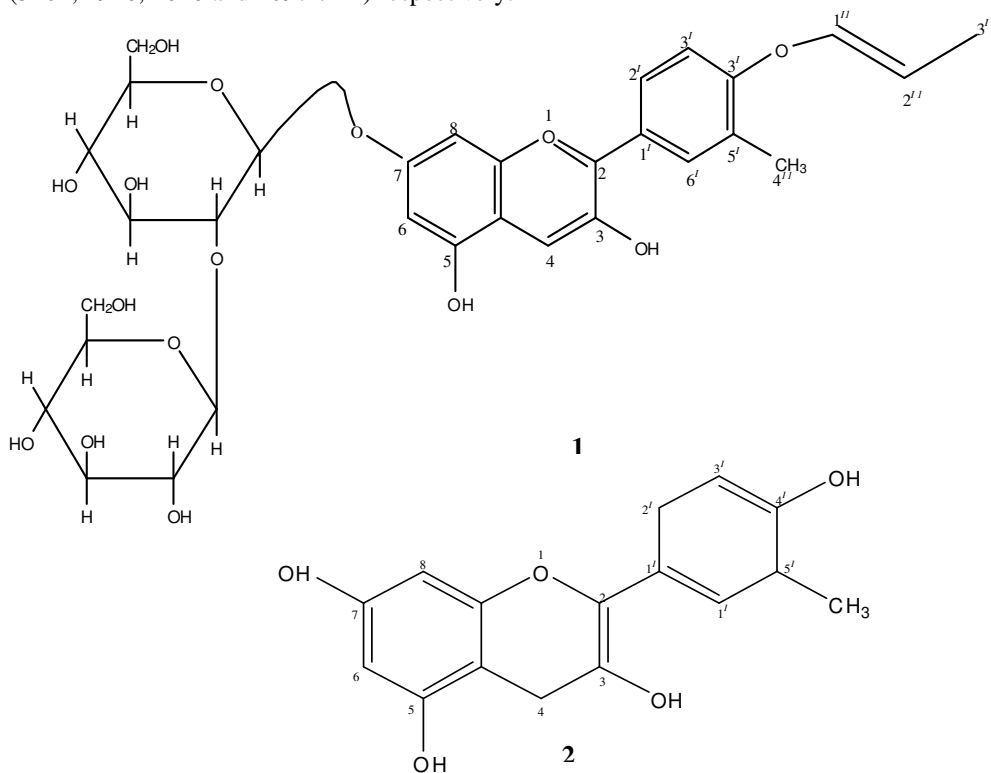
The *in vitro* antibacterial activity of compound **1** was carried out for 24 h culture of five selected bacteria. The bacteria organism used were *Escheria coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Klebsiella pneumonia*. All the test organisms are clinical isolates of human pathogens obtained from the Federal Medical Centre (FMC) Umuahia, Nigeria.

Cultures were brought to laboratory conditions by resuscitating the organism in buffered peptone broth and thereafter agar medium and incubated at 37 °C for 24 h. The antibacterial activity was performed by filter paper disc diffusion technique. The medium (7 g nutrient agar in 250 mL distilled water, autoclaved at 115 °C for 15 min) was cooled to 50 °C. The medium (20 mL) was poured into a sterile Petri-dish and allowed to solidify. It was allowed to stay for 8 h and observed for contamination. The sterility of the medium was tested. 1 g of compound **1** was dissolved in 1 mL of absolute ethanol and made up to 10 mL with distilled water to give a concentration of 100 mg/mL (10% dilution). A colony of each test organism was sub-cultured on nutrient broth and incubated at 37 °C for 8 h. This was then used to flood the agar plates. Sterilized filter paper disc soaked in compound **1** was placed on the plates with test organisms. The plates were incubated at 37 °C for 24 h. After incubation, plates were observed for zones of inhibition (in mm diameter). The minimum inhibitory concentration was determined by comparing the different concentrations of compound **1** having different zones of inhibition and selecting the lowest concentration.

The sensitivity susceptibility of the test bacteria to the standard drug was tested using inoculated agar plate and ciprofloxacin. The zones of inhibition were measured and compared with those of compound **1**.

Results and Discussion

The molecular formula of compound **1** was established as C₃₁H₃₇O₁₅ based on its HREIMS and NMR data. The IR spectrum revealed hydroxyl, aliphatic, aromatic and ether bands at (3401, 2926, 1616 and 1097 cm⁻¹) respectively.



Compound **1** was identified as 5'-methyl 4'-propenoxy anthocyanidine 7-O- β -D-diglucopyranoside was assigned the molecular formula m/z 647.5367 calculated for $C_{31}H_{37}O_{15}$ (m/z 648) with base peak at m/z 57.0709 on the basis of HERIMS. The relative molecular mass of 647.5367 with base peak at m/z 57.0709 confirmed compound **1** as 5'-methyl 4'-propenoxy anthocyanidine 7-O- β -D-diglucopyranoside. The pattern of fragmentation (Figure 1) showed that compound **1** undergoes cleavage or detachment of the propenoxy group C_3H_5O (m/z 57.0709) to produce the base peak. Further fragmentation occurred at C_7 to remove the diglucoside moiety while the detachment at C_4 removes the propen fragment from the anthocyanidine system producing the peak $C_{16}H_{12}O_5$ (m/z 284). Proton migration and hydrolysis produces the peak at (m/z 285.2430) calculated for $C_{16}H_{13}O_5$ (m/z 285). This seems to be a new compound **2**. Also detachment of propenoxy group at C_4' and a methyl group at C_5' from compound **1** afforded the peak at m/z 577.5172 calculated for $C_{27}H_{29}O_{14}$ (m/z 577).

Analysis of the 1H NMR spectrum is shown in Table 1. The 1H NMR spectrum showed the presence of the aromatic protons at δH 7.2609 (1Hs), 7.5155 (1Hs), 7.5380 (1Hs), 7.6938 (1Hs), 7.5158 (1Hs), and 7.5155 (1Hs). The 1H NMR spectrum also contained two methyl signals at δH 0.8803 (3H d) and 0.8962 (3Hs). The protons of the two hydroxyl groups attached to the anthocyanidine nucleus showed broad singlet peaks at δH 4.1213 and 4.1510 respectively. In addition, the olefinic protons showed the signals at δH 5.3360 and δH 5.3266 respectively confirmed by ^{13}C NMR spectroscopy (δc 127.984 (CH) and δc 128.893 (CH).

The ^{13}C NMR chemical shifts for each sugar unit were observed at δc 77.418 (C_1), 77.100 (C_2), 68.987 (C_3), 68.256 (C_4), 76.783 (C_5), and 62.191 (C_6), respectively. The anomeric proton signals were also observed at δH 4.2187, and 4.2832. The anomeric proton signals indicated β -orientation at the anomeric centers. The Compound **1** was identified as propenoxy pelargonidin diglucoside characterized as 5'-methyl 4'-propenoxy anthocyanidine 7-O- β -D diglucopyranoside.

Hydrolysis of Compound **1** produce maltose and pelargonidin derivative characterized as 5'-methyl 4', 3, 5, 7- tetrahydroxy anthocyanidines **2**. The conjugation of the electrons of the heterocyclic ring within the aromatic system may be responsible for the yellow color of the flavonoids. The presence of this phenolic compound indicates that this plant may be antimicrobial agent since phenol and phenolic compounds are extensively used in disinfections and remain the standard with which other bactericides are compared¹². These phenolic compounds in *A. cordifolia* may be responsible for the therapeutic, antiseptic, antifungal or bactericide, as well as anti-viral and anti-tumor activities of *A. cordifolia*³. The bioactive phenolic compounds act as radical scavengers and singlet oxygen quenchers. They react with peroxy radicals and thus bringing about the termination of the radical reaction generated within the system.

The antibacterial activity of compound **1** was evaluated *in vitro*. The results are reported in Table 2. The compound showed a broad diversity regarding growth inhibitory activity (Table 2). The anthocyanidine diglucoside isolated from the leaves of *A. cordifolia* has shown inhibition activity against *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Escherichia coli* (Table 2). This agreed with the findings of Lamikanra *et al.*¹³ who reported that extracts from the leaves of *A. cordifolia* exhibited inhibition on *Staphylococcus aureus* and *Escherichia coli*. The observed inhibiting role on the microorganism explains the reason behind the utilization of *A. cordifolia* extract in traditional medicine as cough suppressant, anti-tumor agent, ulcer treatment and wound healing activity in traditional medicine^{5,7}.

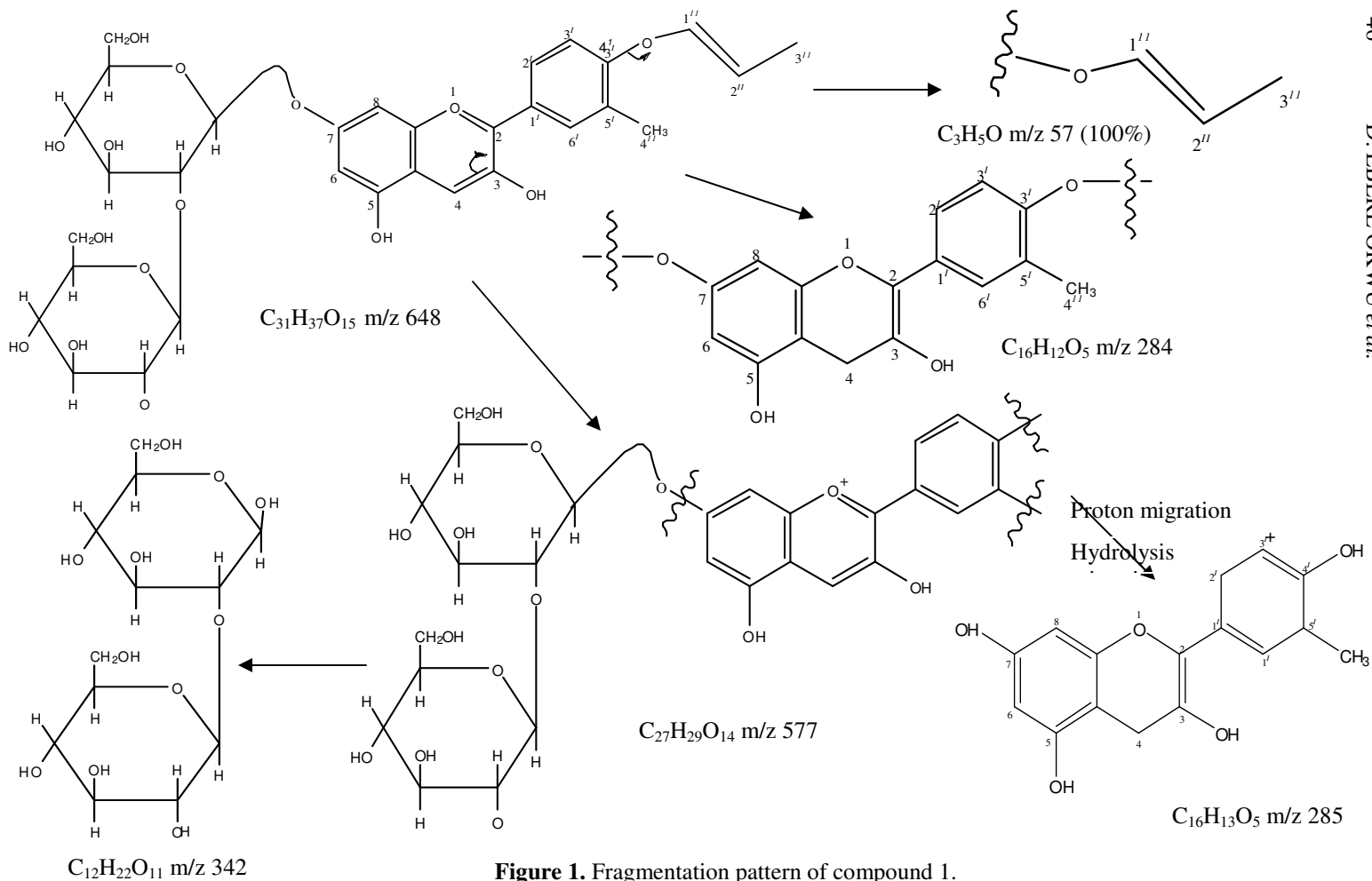


Table 2. Diameter of zones of inhibition (mm) of Compound **1** isolated from *A. cordifolia* leaves and ciprofloxacin

Test organism	<i>A. cordifolia</i> leaf constituents, g/100 g	Ciprofloxacin mg/100 g
<i>Escherichia coli</i>	5.00 ± 0.01	11.00 ± 0.20
<i>Pseudomonas aeruginosa</i>	12.00 ± 0.08	26.00 ± 0.01
<i>Staphylococcus aureus</i>	12.00 ± 0.01	20.00 ± 0.10
<i>Klebsiella pneumonia</i>	11.00 ± 0.01	16.00 ± 0.02
<i>Proteus mirabilis</i>	7.00 ± 0.04	19.00 ± 0.06

Data are means ± Standard deviation of triplicate determination.

These findings shows that phenolic flavonoids are potent water soluble super anti-oxidant and free radical scavengers which prevent oxidative cell damage, have strong anticancer activity and anti-inflammatory properties¹². This bioactive compound isolated is responsible for the marked medicinal properties of the plant. The mechanism of inhibiting action of this phenolic compounds on these microorganism may be due to impairment of a variety of enzyme systems, including those involved in energy production, interference with the integrity of cell membranes and structural component synthesis^{14,15}.

The minimum inhibitory concentration (mic) of Compound **1** on the microorganisms was 6.50-12.50 mg/mL (Table 3). *E. coli*, *P. aeruginosa*, *S. aureus*, *P. mirabilis* and *K. pneumonia* are human commensals and have been incriminated in the infection of wounds^{5,16}. The inhibition of these bacteria justifies the use of *A. cordifolia* in healing wounds, ulcers and toothache in traditional medicine⁵. The ability of this Compound to inhibit these micro-organisms may be the reason behind the use of the leaves of *A. cordifolia* in the treatment of gonorrhoea and urogenital infections in herbal medicine in Nigeria. The isolation and characterization of 5' methyl 4'-propenoxy anthocyanidine 7-O-β-D diglucopyranoside lend credence to the common use of *A. cordifolia* as antibacterial agent. These findings justify the traditional use of this plant for wound treatment in traditional medical practice. The plant offer wide-scope for utilization as raw material by pharmaceutical industries for drug formulation.

Table 3. Minimum inhibitory concentration of Compound **1** Isolated from leaf of *A. cordifolia* on the pathogens, mg/mL.

Test Organism	Concentration of Leaf isolate mg/mL					Mic mg/mL
	100	50	25	12.50	6.50	
	Zone of Inhibition, mm					
<i>Escherichia coli</i>	5.0±0.01	5.0±0.01	4.0±0.01	1.0± 0.01	-	12.50
<i>Pseudomonas aeruginosa</i>	12.0±0.01	7.0±0.01	3.0±0.02	2.0± 0.01		12.50
<i>Staphylococcus aureus</i>	12.0±0.01	9.00±0.02	6.0±0.01	3.0± 0.01	2.0±0.01	6.50
<i>Klebsiella pneumonia</i>	11.0±0.01	5.0 ± 0.03	5.0±0.01	4.0± 0.02	3.0±0.10	6.50
<i>Proteus mirabilis</i>	7.0 ± 0.04	3.0 ± 0.01	3.0±0.01	1.0± 0.01	-	6.50

Data are means of triplicate determinations-No Zone of Inhibition.

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