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Research article

The Isolation Rate of Culturable Actinomycetes from Malaysian Borneo Forests and Their Activity Against Mammalian GSK-3β

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Abstract

More than ten types of forests can be found in Sabah, Malaysian Borneo. Studies comparing culturable actinomycetes potential in this region are relatively scarce. This study described a preliminary statistical comparison of culturable actinomycetes isolation rates and their biological activity against mammalian glycogen synthase kinase-3 (GSK-3β). We isolated 1049 isolates using standard isolation media for actinomycetes (HVA, ISP4 and AIA) with distinctive morphologies from the main forest types in Sabah; primary, secondary, mangrove, and beach forests. Isolate prevalence analysis revealed that secondary forests had the highest soil-to-isolate ratio (1:11). Interestingly, Kruskal-Wallis analysis revealed no significant differences in the overall isolation rates of actinomycetes, including non-sporulating strains, between forest types (P-value=0.142). The crude extracts of these isolates were assayed against GSK-3β, and we identified 19 active isolates; nine from primary and nine from secondary forests (no significant mean difference (P-value=0.558), one from beach forests, and none from mangrove forests. Overall, despite the different sampling locations and soil types, the isolation rates of culturable actinomycetes in Sabah did not vary significantly. However, both primary and secondary forests yielded more actinomycetes isolates that were active against mammalian GSK-3β than mangrove and beach forests. Hence, secondary forests are an attractive alternative to primary forests for exploring bioactive compounds from culturable actinomycetes in Sabah.

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1. Introduction

Natural products play an essential part in the discovery and development of new drugs. Tropical rainforests have been dubbed as the "world's largest pharmacy" and are regarded as the best source for drug discovery, especially from plants and soil microbial communities, which can produce a broad spectrum of organic molecules and complex chemicals [1]. Plants and microorganisms, notably actinomycetes, produce these compounds as a means of communication and defense [2, 3]. In many cases, secondary metabolites from these bacteria also show promising biological activities such as anticancer and antimicrobial effects [4, 5].

The Food and Agriculture Organization (FAO) reported that 620,000 km^2 hectares of primary forests were degraded worldwide from 1990 to 2015. Unfortunately, these data are believed to underestimate the actual number as Indonesia and Venezuela have failed to report to the Forest Resources Assessment in 2015, which was also the case for the Democratic Republic of Congo in 2010 [6]. As of 2017, only 20% of the forests on Earth are Intact Forest Landscapes (IFLs), and only 7.2% are protected [7]. Southeast Asia is facing the most extensive deforestation in the world. According to estimates from imaging satellite observations, between 2000 and 2014, 82,000 km^2 hectares of its primary forests were lost [8]. The ongoing destruction of tropical rainforests has been shown to impact plant, animal species, and microbial communities [9]. Hence, the loss of many undiscovered novel compounds from plants and microorganisms is inevitable.

Sabah, also known as the North Borneo, Malaysia, is one of the world's 12 biodiversity hotspots. More than 60% of Sabah is still covered with forested areas, representing 4.4 million hectares of the landmass. However, less than 8 % of the area is unlogged forest. More than ten types of natural forests can be found in Sabah, including expanding secondary forests [10, 11]. As the loss of natural forests is likely to continue, secondary forests have attracted considerable interest due to their ability to regenerate and lessen biodiversity loss [12, 13]. Although secondary forests are not as rich as primary forests, some studies suggest that primary and secondary forests may have almost equal biodiversity [14, 15]. In addition, coastal forests (mangrove and beach forests) are also prominent forest types in Sabah. Mangrove in Sabah is a productive ecosystem with complex vegetation [16]. Meanwhile, the beach forests of Borneo are characterized by sandy soil dominated by creeping plants with lower diversity that is less suitable to support many species [17].

Many studies have reported the changes in the biodiversity of flora and fauna in different forest types in Southeast Asia [18], but studies on soil microorganisms biodiversity and drug discovery are still scarce. One study examined soil microbial community distribution of five forest types in Borneo, Malaysia, including secondary forest. About 90% of the detected bacterial groups were Acidobacteria, Proteobacteria, Verrucomicrobia, Planctomycetes, and Bacteroidetes. It was further revealed that only 0.2% of species were detected in all forests as the soil microbial community adapts to specific micro-and macro-environments [19]. Our group reported the diversity of Actinobacteria isolated from mangrove forests of Sabah, and they mainly consisted of *Streptomycetales*, *Micromonosporales*, *Mycobacteriales*, and *Microbacteriale* [20]. The taxonomical study between Malaysian (peninsular) and Japanese actinomycete isolates revealed little overlap, with only 14% of the species and 50% of the genera identified from both locations. Interestingly, two strains from Malaysia did not belong to a known genus and were placed in the suborder of *Sreptosporangineae* [21]. Hence, discovering novel actinomycetes mainly distributed in only specific soil in the studied area is possible [22, 23]. It would be interesting to see the effect of different forest types in influencing the isolate species and their secondary metabolites.

In this study, we focused on the most abundant soil microorganisms in the tropics, actinomycetes. While unculturable actinomycetes represent a significant percentage of actinomycetes diversity in soil, they do not grow under standard laboratory settings [24]. Hence, we focus on the isolation rate of culturable actinomycetes using standard isolation media since their

pharmacological potential is still underexplored. We adopted a yeast-based assay system targeting glycogen synthase kinase-3β (GSK-3β) due to its pivotal roles in numerous diseases. We reported the identification of new imides, 3-[(5E)-5-methyl-4-oxo-2-hydroxy-5-octenyl] glutarimide, acting as a selective GSK-3β inhibitors, which were purified from a novel actinomycete strain *Streptomyces* H7667. In addition, non-selective GSK-3β inhibitors, and two cycloheximide derivatives, were also identified from this isolate [25-27]. Recently, we have reported the identification of an antimalarial agent from an actinomycete strain H11809, initially screened against mammalian GSK-3β [28] and a novel actinomycetes strain, closely related to the rare actinomycetes *Kitasatospora aureofaciens*, that inhibited GSK-3β and exerted potent antimalarial activity [29].

GSK-3 is a serine/threonine kinase that is a regulator of numerous signaling pathways in cell, initially shown to phosphorylate glycogen synthase. The abnormal activity level of one of its isoforms, GSK-3β, is known to cause diabetes, cancer, and neurodegenerative diseases in humans [30-32]. In cancer, overexpression of GSK-3β promotes tumor cell growth and is observed in chemotherapy resistance [33]. Hyper-activated GSK-3β also causes hyperphosphorylation of tau, one aspect of Alzheimer's pathology [34]. It is also being examined as an emerging target for protozoan-related disease and microbial infections [35, 36]. Hence, GSK-3β is an exciting target for drug discovery.

Given the previous outcomes from our yeast-based assay targeting GSK-3β and its pharmaceutical potential, this assay was selected to assess the influence of major forest types in Sabah on the isolation rate and biological activity of the culturable actinomycetes. This study provides an insight into the potential of culturable actinomycetes in this region for future work on drug discovery.

2. Materials and Methods

2.1 Isolation of culturable actinomycetes

Soil samples were collected from different forest types in Sabah, Malaysia which were divided into primary, secondary, mangrove, and beach forests. Soil samples were collected at a depth of 10 cm, mostly from under leaf litter. The main focus of this study was to isolate culturable soil microorganisms, especially actinomycetes. Soil samples were heat-treated at 65°C for 15 min, and the dried soil was diluted in a 0.85 % NaCl (w/v) solution (1000-fold) to eliminate non-sporulating bacteria. The main isolation media used in this study was modified humic-acid agar (HVA) with vitamin B. It allows the isolation of actinomycetes genera such as *Streptomyces*, *Streptosporangium, Nocardia, Dactylosporangium, Microtetraspora,* and *Thermomonospora* [37]. In addition, International Streptomyces Project medium no. 4 (ISP4) for the isolation of *Streptomyces* and actinomycetes isolation agar (AIA) for general actinomycetes isolation were also used. Cycloheximide was added to the isolation media at 50 µg/ml to prevent contamination from microfungi. The pH of the isolation media was adjusted at pH 4.5, 7.0, and 9.0 for soil with acidic, neutral, and alkaline pH, respectively.

The isolation plates were incubated for at least 3-4 weeks at 28°C until colonies were observed. Colonies with distinct morphologies were streaked on oatmeal agar (OA) (pH 7.0) to induce sporulation. Morphology observation was carried out and grouped based on aerial and substrate mycelia, pigmentation and sporulation. Sporulation on HVA and oatmeal agar was the main indication that most isolates in this study were actinomycetes.

To further confirm that isolates showing activity against GSK-3β were actinomycetes, 16S rRNA sequences were used. The genomic DNA of the isolates was extracted using a method

described previously [29]. PCR amplification of 16s rRNA was carried out according to modified method previously described with amplifications conditions set at: activation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 55°C for 30 s and 72°C for 30 s, respectively [38]. Sequencing was performed using an ABI Model 3730XL DNA Analyzer. The 16S rRNA sequences were compared to sequences in GenBank using the online service of Basic Local Alignment Search Tool (BLAST).

2.2 Preliminary screening of crude extract against GSK-3β

A single colony of 3-5 days old pure culture was inoculated into mannitol-peptone broth (10 ml) and incubated at 28°C, 220 rpm for three days as seed culture. It was then seeded into production medium (30 ml mannitol-peptone broth) at 1% (v/v) and was further incubated for five days. An equal volume of acetone was added after the incubation period and left overnight at room temperature. The crude extracts were then filtered using Whatman filter paper No.1 to remove the cell debris. The samples were freeze-dried and re-dissolved in methanol to prepare 100 mg/ml of crude extracts.

The activity of the crude extracts against mammalian GSK-3β expressed by genetically modified yeast strains was performed using disc diffusion assay, as described previously [28]. Briefly, the modified yeast strain was grown in 5-10 ml SC -ura broth, incubated at 28°C, 180 rpm for two days. To perform the screening, 1.0 ml of the yeast seed culture was inoculated into 100 ml SC -ura agar for disc diffusion assay. The extracts were tested at 2 mg/ml using *Streptomyces* H7667 crude extracts (GSK-3β inhibitor producer) as positive control and methanol as negative control. The plates were incubated at 28°C (allowing temperature) and 37°C (defecting temperature) for five days, which daily observation performed to measure the zone of inhibition (ZOI, mm) was performed [39, 40].

2.3 Data analysis

2.3.1 Isolate prevalence based on sampling locations

The isolate prevalence was calculated to evaluate the percentage of isolating at least one soil bacterial strain from a pre-treated soil sample in this study with distinctive morphology (1).

$$\text{Isolate prevalence} = \frac{\text{Total number of sample yield} \geq \text{one distinctive isolate x } 100}{\text{Total number of soil samples}} \quad (1)$$

2.3.2 Kruskal-Wallis analysis

A Kruskal-Wallis analysis was performed using SPSS software. This test is a modified version of the analysis of variance (ANOVA) that is commonly used to analyze non-normally distributed data [41]. The significance level of this test is similar to that of one-way ANOVA (95% confidence level).

2.3.3 One-tailed t-test

An independent *t*-test (SPSS software) was performed to evaluate the mean differences in isolating potential isolates between primary and secondary forests.

3. Results and Discussion

The distribution of soil actinomycetes is highly associated with organic materials that are made up of plant and animal parts at different degradation stages [42]. Hence, it was anticipated that most actinomycete isolates would likely be isolated from both primary and secondary forests. Dense plant growth provides abundant organic materials for the soil organic matter (SOM) cycle that provides adequate nutrients for microorganism distribution, in contrast to less dense forests, such as coastal forests [43]. Thus, soil samples in this study were collected around the roots of different plants.

A total of 368 soil samples were collected from different forest types in Sabah, and 1049 actinomycete strains were isolated. Soil pre-treatment applied in this study significantly reduced the number of non-sporulating soil microorganisms. High temperature and salinity were reported to prevent the growth of non-filamentous bacteria and are effective for the isolation of rare actinomycetes, including genus *Micromonospora*, *Dactylosporangium,* and *Streptosporangium* [44, 45]. We observed that the growth of soil microorganisms on the three different media used in this study was significantly different as more actinomycete colonies were isolated on HVA than AIA and ISP4. HVA is more effective for actinomycetes isolation as it induces their sporulation for easy selection of actinomycete colonies while restricting the growth of non-filamentous bacteria colonies due to the presence of humic acid as the sole carbon source. Meanwhile, most colonies observed on ISP4 and AIA were non-sporulating.

In this study, most of the sporulating isolates were isolated from secondary forests (63.0%), in which brown and grey spores were the most abundant, representing 24.5% and 23.4% of the total sporulating isolates, respectively. Other colors observed were black and blue, at 4% each. Most isolates from mangrove and beach forests were non-sporulating on OA (Table 1).

Table 1. The observation of actinomycetes and other soil microorganisms (non-sporulating) based on the color of aerial mycelium and spore

Color	Secondary forests		Mangrove forests		Beach forests		Total/Percentage	
	S	NS	S	NS	S	NS	S	NS
Grey	106	1	3	0	11	0	120 (23.4%)	1 (0.2%)
Brown	115	2	6	0	5	2	126 (24.5%)	4 (0.8%)
White	50	44	1	16	9	23	60 (11.7%)	83 (16.2%)
Green	14	0	1	0	2	0	17 (3.3%)	0 (0.0%)
Yellow	9	19	2	21	2	8	13 (2.5%)	48 (9.3%)
Red	13	0	0	0	0	0	13 (2.5%)	0 (0.0%)
Orange	9	2	0	7	0	1	9 (1.8%)	10 (2.0%)
Blue	4	0	0	0	0	0	4 (0.8%)	0 (0.0%)
Black	4	2	0	0	0	0	4 (0.8%)	0 (0.0%)
Total/ Percentage	324 (63.0%)	70 (13.6%)	13 (25.3%)	44 (8.7%)	29 (5.6%)	34 (6.6%)	366 (71.2%)	148 (28.8%)

S = Sporulating on OA, NS = Non-sporulating on OA

3.1 Isolate prevalence analysis and Kruskal-Wallis analysis

Secondary forests had the highest isolate prevalence (82.6%), followed by mangrove forests (82.3%), primary forests (77.0%), and beach forests (17.3%). On average, 11 strains of actinomycetes were isolated from each soil sample collected from secondary forests in this study (1:11). Soil-to-isolate ratios of the other forests were 1:5, 1:4, and 1:3 for beach, mangrove, and primary forests, respectively (Table 2). This suggested that soil from secondary forests had the highest probability of isolating culturable actinomycetes. Surprisingly, primary forests had the second-lowest soil-to-isolate ratio and isolate prevalence, which was even lower than mangrove forests. Kruskal-Wallis analysis further revealed that the number of culturable actinomycetes

isolates in this study was not significantly influenced by forest type (H (4) = 6.887, p = 0.142), with a mean rank of 44.53 for primary forests, and 52.62, 45.76, and 31.65 for secondary forests, mangrove forests, and beach forests, respectively. However, multiple post-hoc comparisons analysis showed a significant isolate prevalence difference between secondary and beach forests (H (1) = 4.873, p = 0.027), with a mean rank of 21.18 for secondary forests and 13.82 for beach forests. These results further supported the observation that secondary forests had the highest isolate prevalence in this study (Table 3).

Table 2. Statistical analysis of soil samples and isolates from different type of forests

Type of forest	Sampling site	Total of soil sample[1]	No. of soil produces at least one isolate	No. of isolate[2]	Isolate prevalence (%)[a]	Soil to no. of isolates ratio[b] (soil:isolate)
Primary	Danum Valley	217	167	535	77.0	1:3
Secondary	Crocker Range Likas Kudat Lahad Datu Membakut	46	38	394	82.6	1:11
Mangrove	Klias Kalabakan	17	14	57	82.3	1:4
Beach	Mantanani Karambunai	81	14	63	17.3	1:5
Total		368	234	1049		

[a] indicates the percentage of soil that produces at least one isolate from the respective sampling sites.

[b] indicates the average of isolates that can be isolated from each soil from the respective sampling sites, expressed as the ratio of "1" and "2".

Table 3. Multiple comparisons of forest types based on mean difference using Kruskal-Wallis analysis

Post-hoc comparison		p-value
Primary forests	Secondary forests	0.271
	Mangrove forests	0.806
	Beach forests	0.070
Secondary forests	Primary forests	0.271
	Mangrove forest	0.313
	Beach forests	0.027*
Mangrove Forests	Primary forests	0.806
	Secondary forests	0.313
	Beach forests	0.075
Beach forests	Primary forests	0.070
	Secondary forests	0.027*
	Mangrove forests	0.075

* indicates a significant difference based on 95 % of the significance level

Actinomycetes are known to establish a symbiotic interaction with plants, mainly in the rhizosphere zone [46]. This zone is directly influenced by plants as up to 60% of the photosynthetic products are secreted from the roots, such as amino acids, sugars, protein, and secondary metabolites. The rhizosphere zone creates a complex microenvironment for actinomycetes [47]. Hence, root niches can be created by particular plant species that may boost specific soil microorganism groups.

Furthermore, soil organic matter (SOM) fractions are available at different sizes and degrees of degradation in the soil. Disturbances in land use, such as cropping and plowing, can alter the SOM fraction sizes. Lighter or smaller SOM fractions can decompose faster than coarse organic matter [48]. Secondary forests are reclaimed forest that has been previously disturbed in some ways, either by natural causes (natural disasters) or unnatural causes (such as logging or agriculture). Bursts of microbial activity and decomposition rates of SOM that enriched the labile fraction were previously reported in secondary or disturbed forests [49-51]. Hence, the high soil-to-isolate ratio and soil prevalence of secondary forests in this study can be due to higher SOM in the soil compared to other forest types.

Primary forests are recognized as the most biodiverse ecosystems on earth as they are the habitats of numerous floral species that cannot be found in other types of forests [52, 53]. A genetic study revealed that the actinomycete diversity of primary forests is richer than secondary forests. In addition, primary forests were also indicated to have a higher percentage of unculturable or rare actinomycetes, such as *Sphaerisporangium* and *Actinophytocola* [54, 55].

Although HVA media was utilized to isolate rare culturable actinomycetes in this study, the associated plant microbiome community from Bornean primary forests may require specific media or soil pre-treatments for their isolation. It is recognized that root exudates are diverse among different species, ages, and plants [56]. In general, floral endemism in Malaysian Borneo is estimated to be almost 50% (or 8,000 plant species) [57]. Thus, the low soil-to-isolate ratio of primary forests in this study might be due to the presence of rare unculturable actinomycetes, contributed by a diverse endemic species of plants in primary forests of Sabah.

Mangrove and beach forests are usually categorized as coastal forests. In this study, these forests were analyzed separately due to their apparent soil-type differences. Soil texture may lead to the variability of the SOM in the soils. Soils in mangrove forests are generally made up of sand, silt, clay, and mud. It is interesting to note that although mangrove soil is rich in organic matter, the top soils of mangrove forests are minimally aerated, which causes a lower decomposition rate of organic material and thus minimal microbial distribution [58].

Beach forests are known for their high soil porosity and their inability to hold water. Sandy soils of beach forests are expected to have the lowest SOM among forest types due to the extreme abiotic factors of temperature and moisture, which affect the accumulation of organic matter. Moreover, litter inputs for decomposition are also lower because of limited primary sources as the plants on beach forests are limited to certain species, such as *Casuarina equisetifolia* [59]. Hence, beach forests had the least isolates in this study.

3.2 Biological activity of the isolated strains against GSK-3β and their statistical analysis

The inhibitory activity towards GSK-3β was selected as an indicator for comparison due to its significant involvement in various diseases. A total of 19 isolates that produced GSK-3β inhibitors were detected from a yeast-based screening assay. Primary and secondary forests yielded the most potential isolates (9 isolates each), while beach forests yielded one active isolate and none from mangrove forests. Based on the results, all the active isolates were culturable actinomycetes (based on spore morphology on OA). Their crude extracts exerted ZOI more significantly at 37°C (varying

diameters) than ZOI at 28ºC (with diameter less than 8 mm), indicating possible selective inhibition of GSK-3β (Table 4). In addition, 21 crude extracts with toxic activity were also identified in this study.

Table 4. Potential isolates and their morphological observation

Sampling sites	Isolate	Aerial mycelium color	Substrate mycelium color	Extracellular pigmentation	Anti-GSK-3 activity
Primary forest	H11809	Light brown	White	No	+++
	H11490	Grey	Yellowish orange	No	++
	H11526	Red	White	No	++
	H11695	White	Orange	No	++
	H11785	White	Light yellow	No	++
	H11462	Greyish brown	Yellowish grey	No	++
	H11720	White	White	No	++
	H11668	Violet	Dark purple	No	+
	H11686	Grey	Light grey	No	+
Secondary forest	FA013	Grey	Brown	Yellow	+++
	FH025	Dark grey	Reddish-brown	Brown	+++
	H17(1)-2	Grey	Grey	No	+++
	H17(2)-1	White	White	No	+++
	TA1528	Grey	Yellowish-brown	No	++
	TA1529	Red	Yellowish-brown	No	++
	SM0307	Black	Dark brown	No	++
	FH038	Greenish brown	Light brown	Brown	+
	TA1522	Grey	Yellowish-brown	Yellow	+
	FH039b	Brown	Brown	No	+
Beach forest	MI4701	Brown	Brown	No	++

+++ = Most potent (ZOI ≥ 15 mm at 37ºC)
++ = Moderate (ZOI ≥ 10 mm at 37ºC)
+ = Weak (ZOI ≥ 8 mm at 37ºC)

The mutant yeast strain used in this study was constructed by removing yeast homolog *GSK-3* genes to produce a mutant clone unable to grow at a higher temperature (>37 ºC). However, the insertion and expression of mammalian GSK-3β by the mutant clone, restores its original phenotype. Hence, the specific inhibition of GSK-3β causes growth defects only at 37ºC. Furthermore, the yeast-based assay applied in this study was previously shown to be selective towards GSK-3β inhibitor targeting Cys-199 on the ATP-binding site such as TDZD-8 and staurosporine [40]. Due to this specificity of the assay, only 19 potential isolates were identified.

An independent-samples *t*-test was conducted to compare the activity of actinomycetes isolated from primary and secondary forests against GSK-3β. There was no significant difference in the activity against GSK-3β for primary forests (M = 1.97, SD = 0.173) and secondary forests (M = 1.96, SD = 0.203); t (324) = 0.587, p = 0.558. This data suggests that the probability of isolating potential actinomycete from both primary and secondary forests is equal (Table 5).

Table 5. T-test analysis comparing primary and secondary forests on the activity of the isolates against GSK-3β

Forest type	n	Mean	SD	t-cal	t-crit	df	p	Decision
Primary	163	1.97	0.173	0.587	1.65	324	0.558	No significant
Secondary	163	1.96	0.203					difference

The production of secondary metabolites by actinomycetes results from interaction with other organisms, such as plants (plant-root microbiome), microorganisms, insects, and animals [60]. *Arabidopsis thaliana* is a model plant used to study the influence of root exudates on the distribution and secondary metabolite production by actinomycetes. This plant exudes more than 100 molecules into its rhizosphere. One of the most significant molecules is jasmonic acid, which favors Streptomyces species' distribution and antimicrobial production. However, it is unknown whether the antimicrobial production by *Streptomyces* in the presence of jasmonic acid is due to a stress response or due to the evolutionary symbiotic response between *A. thaliana* and *Streptomyces* [61, 62].

Besides roots exudates, plants also enrich their surrounding soil by the degradation of plant parts and secretions. Dipterocarp species are among the most commonly found trees in primary and secondary forests in the study areas [63, 64]. Dipterocarp seeds and resins have high phenolic and tannin contents, which are defensive chemicals [65, 66]. Phenolic compounds are usually toxic to soil microorganisms, especially to the non-actinomycetes, as they alter the integrity, fluidity, and conformation of their membrane proteins [67].

Some actinomycetes, especially *Micromonospora*, are known to be resistant to phenolic compounds. Selective pressure has led them to produce novel secondary metabolites to detoxify phenols [68]. Overall, more than 90% of plant species from Borneo's primary forests can also be found in its secondary forests [69]. Thus, the bioactivity of the cultivable actinomycetes from these forests would probably be similar, as suggested by the equal potential of actinomycetes to produce GSK-3β inhibitors in this study.

4. Conclusions

The findings in this study suggest that secondary forests have a potential equal to primary forests in both the isolation rate of culturable actinomycetes and their activity against GSK-3β. Hence, secondary forests are attractive sampling sites for drug discovery from culturable actinomycetes in Sabah.

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