Antimicrobial Activity of Chlororinated Bibenzyl Compounds

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Chlororinated bibenzyl compounds (1 and 2) inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (2 mm inhibition zone and 2 mm inhibition zone at 30 μg/disc), *Candida albicans* ATCC 14053, (2 mm inhibition zone and 2 mm inhibition zone at 30 μg/disc), and the dermatophytic fungi *Trichophyton mentagrophytes* ATCC 28185, (3 mm inhibition zone and 7 mm inhibition zone at 30 μg/disc) and *Cladosporium resinae* ATCC 52833 (1 mm inhibition zone at 30 μg/disc).

Key words : Chlororinated bibenzyl compounds (1 and 2), *Bacillus subtilis*, *Candida albicans*, *Bacillus subtilis*, *Trichophyton mentagrophytes*, *Cladosporium resinae*, antifungal activity

Introduction

Riccardia marginata (Colenso) Pearson (family Aneuraceae) is endemic to New Zealand[5], which it is found growing on old logs in wet forests[1]. No previous chemistry of *R. marginata* has been reported, but the genus *Riccardia* has yielded a range of compounds[2], including sesquiterpene-linked phenol derivatives[3,4], indole alkaloids[5,6] and bis-bibenzyls[7-9]. Brominated bibenzyls have previously been found in the red alga *Polysiphonia urceolata*[10]. The ether extract of the New Zealand liverwort *Radula marginata* isolated a new cannabinid type bibenzyl compound named perrottetinenic acid, and two new bibenzyls, together with a known cannabinoid, perrottetinene. The structure of perrottetinenic acid was a similar to that of delta-1-tetrahydrocannabinol, a known hallucinogen[11]. A crude extract of one of our collections of *R. marginata* showed activity against *Bacillus subtilis, Candida albicans* and *Trichophyton mentagrophytes* in our antimicrobial screening[12]. The chlororinated bibenzyl compound (1) inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (2 mm inhibition zone at 30 μg/disc), Candida albicans ATCC 14053, (2 mm inhibition zone at 30 μg/disc), and the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (3 mm inhibition zone at 30 μg/disc)[13]. We now report the chlororinated bibenzyl compounds (1 and 2) as the antifungal compounds from *R. marginata.*

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Materials and Methods

1. General experimental procedures.

Solvents for extraction and chromatography were distilled prior to use. Preparative silica gel TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254}, visualized with an UV lamp then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) and heating. UV spectrum was recorded with a Jasco V-550 UV spectrophotometer. IR spectrum was obtained with a Perkin-Elmer 1600 FTIR as a film on a NaCl disk. NMR spectra were recorded at 25℃ on a Varian INOVA 500 NMR spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C, using solvent signals as references ($CHCl_3$ at 7.25 ppm, $CDCl_3$ at 77.0 ppm). DEPT, HSQC, CIGAR and NOESY experiments were run at 25℃. EIMS was obtained on a VG70-250S double-focusing magnetic sector mass spectrometer. Column chromatography used octadecyl-functionalized sillica gel (Aldrich C_{18}) and 40-63 μm silica gel 60 (Merck). TLC was carried out on silica gel F254 plates (Merck), with the solvent system hexane - ethyl acetate (9 : 1).

2. Plant materials.

Riccardia marginata(*R. marginata*) was collected from New Zealand's sub-Antarctic Auckland Islands in January 1994 (OTA046755), and near the Kiahoka Lakes, Cape Farewell, West Coast, New Zealand (OTA 050850) in March 1999. Collections were identified by R. Tangney and voucher specimens are deposited in the University of Otago Herbarium.

The samples were air-dried (30℃).

3. Extraction and isolation.

The remaining plant material (46.05 g) from the two *R. marginata* collections was combined, extracted with $CHCl_3$ (4 x 460 mL), and concentrated under vacuum to give a deep green solid residue (1.67 g). The residue was chromatographed on C18 using a steep, stepped solvent gradient from H_2O to MeOH to $CHCl_3$ to hexane. The fraction that eluted at 100% MeOH (221 mg) was chromatographed further on silica gel, eluting with hexane - ethy acetate mixtures. The first two fractions (14 mg), eluted between hexane and 10% ethyl acetate, were further purified by silica gel TLC (plate thickness, 0.2 mm) using 10% ethy acetate in hexane (developed twice, drying the plates between) to give 2′-chloro-3′-hydroxybibenzyl (1, 1 mg, R_f 0.25): yellow gum; UV (MeOH) λ_{max} (log ε) 212 (4.0), 276 (3.2), 282 (3.1) nm; IR (film) ν_{max} 3523, 2917, 2845, 1580, 1450, 1292, 1190, 788, 757, 699 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ (1H, 7.20 m, 2-H), (1H, 7.28 m, 3-H), (1H, 7.21 m, 4-H), (1H, 7.28 m, 5-H), (1H, 7.20 m, 6-H), (2H, 2.89 m, α), (2H, 3.00 m, α′), (1H, 6.88 dd, J=1.5, 8.0 Hz, 4′-H), (1H, 7.07 t, J=7.5Hz, 5′-H), (1H, 6.74 dd, J=1.5, 7.5 Hz, 6′-H), (OH, 5.61 s,); ^{13}C-NMR ($CDCl_3$) δ 140.0 (C-1), 128.4 (C-2), 128.4 (C-3), 126.1 (C-4), 128.4 (C-5), 128.4 (C-6), 36.0 (C-α), 36.0 (C-α′), 141.3 (C-1′), 119.8 (C-2′), 151.6 (C-3′), 113.8 (C-4′), 127.5 (C-5′), 122.0 (C-6′); EIMS *m/z* 235 (1), 234 (7), 233 (3), 232 [M^+] (18), 141 (15), 91 (100); HREIMS *m/z* 232.0651 (calcd for $C_{14}H_{13}ClO$, 232.0655). 2′,4′,6′-trichloro-3-hydroxybibenzyl (2, 3 mg, R_f 0.24): yellow gum; UV (MeOH) λ_{max} (log ε) 209 (4.3), 279 (3.1), 286 (3.2) nm; IR (film) ν_{max} 3380, 2940, 2869, 1559, 1500, 1452, 1422, 1315, 1291, 1273, 1226, 1148, 805, 770, 741, 729, 688 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ (1H, 7.30 m, 2-H), (1H, 7.25 m, 3-H), (1H, 7.22 m, 4-H), (1H, 7.25 m, 5-H), (1H, 7.30 m, 6-H), (2H, 2.82 m, α), (2H, 3.15 m, α′), (1H, 7.33 s, 5′-H), (OH, 5.86 s,); ^{13}C-NMR ($CDCl_3$) δ 137.1 (C-1), 128.5 (C-2), 128.4 (C-3), 126.3 (C-4), 128.4 (C-5), 128.5 (C-6), 34.1 (C-α), 34.0 (C-α′), 140.9 (C-1′), 122.0 (C-2′), 147.0 (C-3′), 118.8 (C-4′), 128.2 (C-5′), 125.8 (C-6′); EIMS *m/z* 304 (2), 303 (1), 302 (8), 300 [M^+] (9), 211 (5), 209 (5), 91 (100); HREIMS *m/z* 299.9889 (calcd for $C_{14}H_{11}Cl_3O$, 299.9875).

4. Screening for antiviral activity

The compounds were applied (30 μL of a 5 mg/mL solution) to a small filter-paper disc, dried, and assayed for antiviral activity using Schroeder et al., methods[14]. The results were observed either cell death (cytotoxicity), inhibition of virus replication, no effect (i.e., all of the cells show viral infection), or a combination of all three. The results were noted as the approximate size of the circular zone, radiating from the extract sample, from 1+ to 4+ representing 25% through to whole well sized zones. The notation used is inhibition/antiviral activity. The type of antiviral effect, indicated by a number after the size of the zone, was also considered important and may give some indication as to the mode of cytotoxic action.

5. Screening for antibacterial and antiyeast activities

Activity against the following bacterial strains and yeast was tested: multiresistant *Bacillus subtilis* (ATCC 19659), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Candida albicans* (ATCC 14053) and *Cladosporium resinae* (ATCC 52833). Compounds were dissolved and diluted in an appropriate solvent (usually ethanol : water) to a concentration of 5 mg/mL. Test plates are prepared from Mueller Hinton agar containing extract to give a final concentration of 100 μg extract/mL agar. Activity growing cultures of the test strains were diluted in saline so as to deliver 10^4 colony forming units onto the test, control (solvent), and blank (agar only) plates with a multipoint inoculator. Inoculated plates were incubated overnight at 37℃. Growth on the blank and control plates was checked and, if satisfactory, growth on the test plates was scored for each test strain as follows: (-) inhibition, no reduction in growth compared with the control, (+) inhibition, no growth. Solutions of compound for assay were dried onto 6 mm filter paper disks, which were then placed onto seeded agar Petri dishes and incubated. Activity was observed as a zone of inhibition around the disk, with its width recorded from the edge of the disk in mm. HM and SM refer to the observed margin surrounding the zone of inhibition. (H= hazy, S= sharp).

6. Screening for antifungal activity

Activity against the following fungal strain was tested: *Trichophyton mentagraphytes* (ATCC 28185) local strain]. Fungal spore suspensions of the test oraganisms were applied to dextrose agar plates. Aliquots of the compound solutions were applied to filter paper discs, at 30 μg extract/disc, and dired at 37℃ for two hours. These discs were applied to the agar plates, two per plate, and incubated at 28℃.

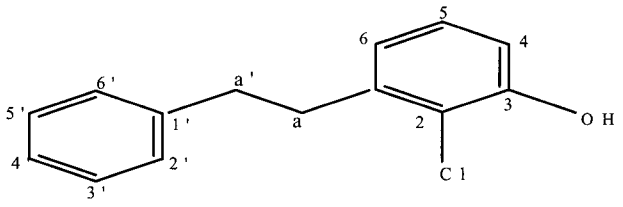
7. Screening for cytotoxic activity

This is a measure of the ability of a sample to inhibit the multiplication of murine leukaemia cells. The sample was dissolved in a suitable solvent, usually ethanol, at 5 mg/mL, and 30 μL of this solution was placed in the first well of a multiwell plate. Seven two-fold dilutions were made across the plate. After addition of the cell solution, the concentration range in the test wells was 25,000 down to 195 ng/mL. After

incubation for three days, the plates were read using an ELISA palte reader at 540 nm wavelength. Automated reading of the plates was possible with the addition of a MTT tetrazolium salt (yellow color). Healthy cells reduce this salt to MTT formazan (purple color).

Results and Discussion

A crude extract of *R. marginata* inhibited the growth of the Gram-positive bacteria and fungus of the extract prepared from *R. marginata*. The major low-polarity secondary metabolites 1 and 2 were purified by chromatography on reversed-phase C18 followed by normal-phase silica[12,13]. The ^{1}H-NMR spectra of these compounds showed -CH_2-CH_2- signals (4H multiplets, δH 2.82 - 3.15 ppm) and phenolic OH signals (D_2O - exchangeable broad singlet, δH 5.61 - 5.86 ppm). The other signals were of aromatic protons, which were overlapping, especially for the minor compound (1). Negative electrospray ionization (-ESI) MS gave rather confusing result for 1 and 2, but did suggest the presence of chlorine atom. Electron-impact MS gave molecular ions corresponding to $C_{14}H_{13}ClO$ for 1 and $C_{14}H_{11}Cl_3O$ for 2. All these spectra showed strong *m/z* 91 ions, due to $C_6H_5CH_2^+$. These results, along with the NMR data above, showed that theses compounds were two bibenzyls, each with one unsubstituted ring and one ring substituted with -OH and -Cl (s). The ^{1}H-NMR spectrum of the monochloro compound (1) was best dispersd and showed a three-proton spin system due to a 1,2,3-trisubstituted aromatic ring. The shielded position of one of the CH signals (δ_H 6.89, δc 113.8) suggested that this was *ortho* to the OH, with another CH signal (δ_H 6.74, δc 122.0) showing NOESY correlations to the -CH_2-CH_2-bridge protons, giving structure 1. This was confirmed by 2D-NMR correlations (Fig. 1) and by ^{1}H and ^{13}C shifts very similar to the known compound bitungolide A (3)[15].



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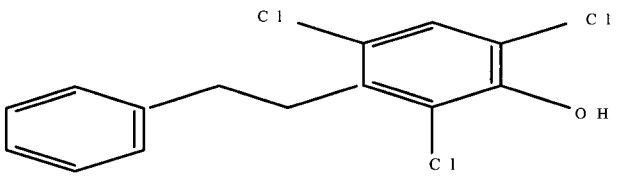

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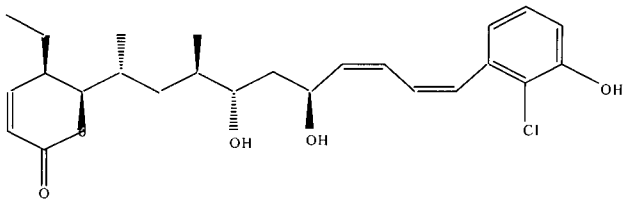

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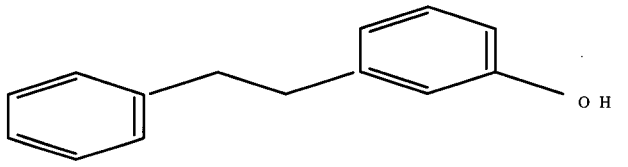

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Fig. 1. The structures of 2-chloro-3-hydroxybibenzyl (1) 2,4,6-trichloro-3-hydroxybibenzyl (2), bitungolide A (3) and 3-hydroxybibenze (4)

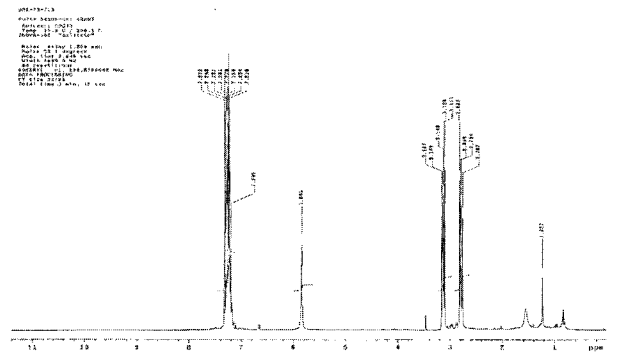

Fig. 2. ^{1}H-NMR Spectrum of 2,4,6-trichloro-3-hydroxybibenzyl(2)

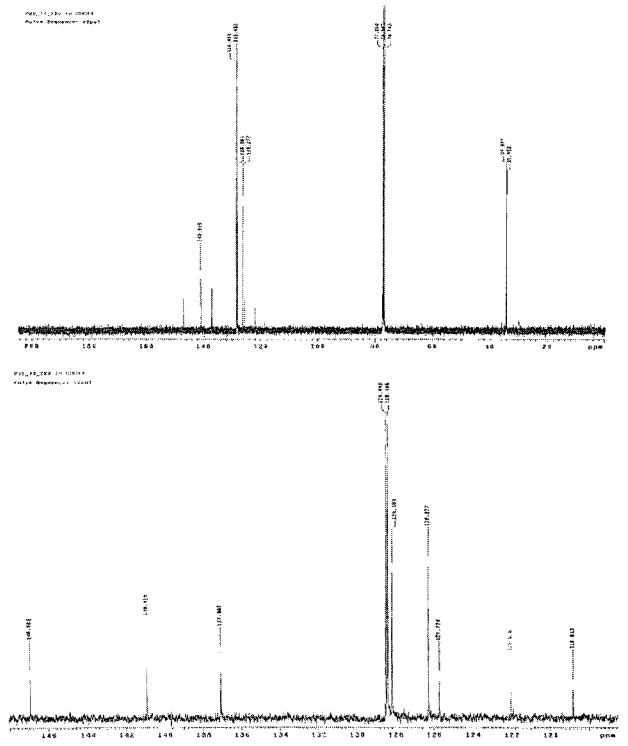

Fig. 3. ^{13}C-NMR Spectrum of 2,4,6-trichloro-3-hydroxybibenzyl (2).

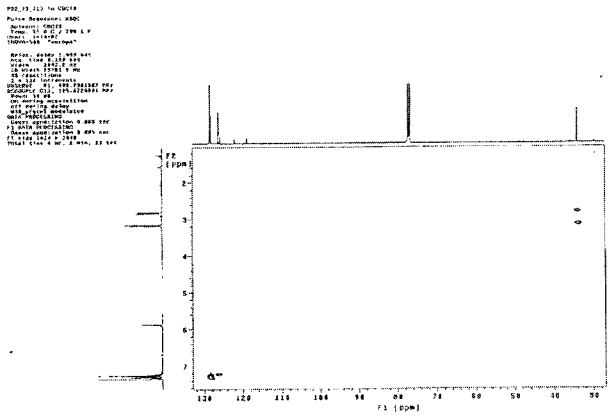

Fig. 4. HSQC Spectrum of 2,4,6-trichloro-3-hydroxybibenzyl (3).

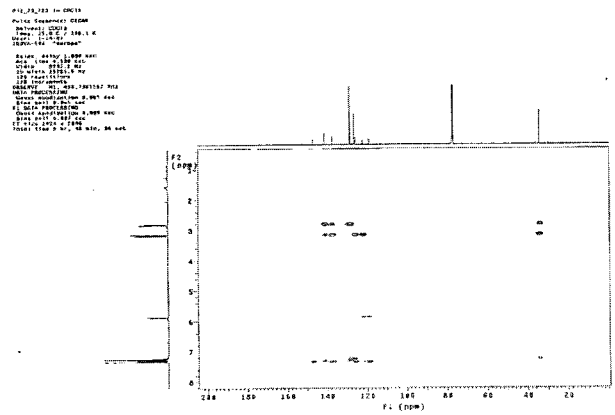

Fig. 5. CIGAR Spectrum of 2,4,6-trichloro-3-hydroxybibenzyl (2).

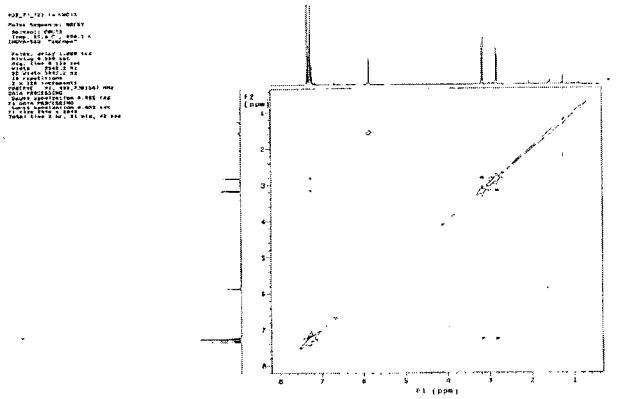

Fig. 6. NOESY Spetrum of 2,4,6-trichloro-3-hydroxybibenzyl (2).

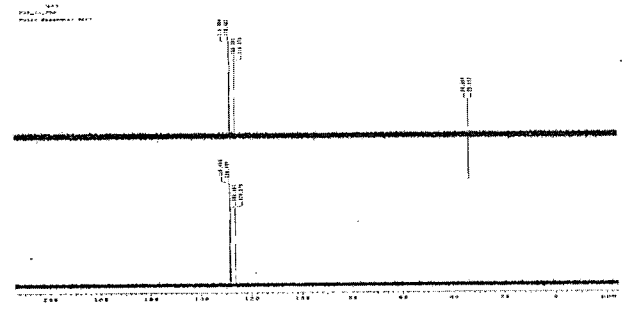

Fig. 7. DEPT Spectrum of 2,4,6-trichloro-3-hydroxybibenzyl (2)

The monochloro compound 1 gave mainly $[M_2 - H]^-$ and trichloro compound 2, with bulky chlorines flanking the OH, showed mainly $[M - H]^-$ with almost no association to give $[M_2 - H]^-$. The trichloro compound 2 showed a singlet for a CH with no NOE interactions with the $-CH_2-CH_2-$ bridge protons or the OH. 2D-NMR correlations supported structure 2. This compound (1) seem to represent successive chlorination of bibenzyl (4) by a haloperoxidase, similar to that recently found in the liverworts *Caldariomyces fumigo* and *Bazzania trilobata*[16] .3-Hydroxybibenzyl (4) has been rerported once as a natural product, in another liverwort, Radula frondescens[2], but we did not see NMR signals appropriate for 4 in any chromatographic fractions or in the crude extract of *R. marginata*.

The *R. marginata* collection containing 1 gave an extract with antimicrobial activity against the Gram-positive bacterium *Bacillus subtilis* and the dermatophytic fungus *T. mentagrophytes*, whereas the Auckland Islands extract was inactive. Assay of 1 showed activity against *B. subcillus*, *T. mentagrophytes*, and the yeast *C. albicans*, but not against the Gram-negative bacteria *E. coli* and *P. aeruginosa*. and against the plant pathogenic fungus *C. resinae*(Table 2).

Table 1. Antimicrobial activity of chlororinated bibenzyl compounds (1 and 2) from *R. marginata*.

Tested	Antimicrobial activity[a]					
material	*B. subtilis*	*C. albicans*	*T. ment.*	*E. coli*	*C. resinae*	*P. aeruginosa*
1	HM 2	HM 2	HM 3	-	-	-
2	HM 2	HM 2	HM 7	-	HM 1	-
Chloramphenicol	SM 13	0	0	0	0	0
Nystatin	0	SM 12	SM 6	0	SM 10	0
Gentamycin	0	0	0	SM 9	0	SM 10

[a]Width of zone of inhibition in mm: 30 μg/disc; -: not detected, 0: not determined. Chloramphenicol: 30 μg/disc, Gentamycin: 30 μg/disc, Nystatin: 100 unit/disc. HM: Hazy margin, SM: Sharp margin, numbers refer to zone of inhibition (mm)

Table 2. List of microorganisms used for antimicrobial susceptibility test.

Gram-positive bacterium	
Bacillus subtilis	ATCC 19659
Gram-negative bacteria	
Escherichia coli	ATCC 25922
Pseudomonas aeruginosa	ATCC 27853
Fungi	
Cladosporium resinae	ATCC 52833
Candida albicans	ATCC 14053
Trichophyton mentagrophytes	ATCC 28185

As indicated in Table 1, chlororinated bibenzyl compounds (1 and 2) inhibited the growth of the Gram-positive bacterium *B. subtilis* ATCC 19659, (2 mm inhibition zone) at 30 μg/disc. Antiyeast activity was observed against the fungus *C. albicans* ATCC 14053 (2 mm inhibition zone) at 30 μg/disc. Antifungal activity was shown against the dermatophytic fungus *T. mentagrophytes* ATCC 28185, (3 mm and 7 mm inhibition zones) at 30 μg/disc. However, 2,4,6-trichloro-3-hydroxy bibenzyl (2)

inhibited the growth of the fungus *C. resinae* ATCC 52833 (1 mm inhibition zone) at 30 μg/disc. These compounds (1 and 2) showed weaker antimicrobial activity than reference compounds (Tables 1 and 2).

In conclusion, chlororinated bibenzyl compounds (1 and 2) inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (2 mm inhibition zone and 2 mm inhibition zone at 30 μg/disc), *Candida albicans* ATCC 14053, (2 mm inhibition zone and 2 mm inhibition zone at 30 μg/disc), and the dermatophytic fungi *Trichophyton mentagrophytes* ATCC 28185, (3 mm inhibition zone and 7 mm inhibition zone at 30 μg/disc) and *Cladosporium resinae* ATCC 52833 (1 mm inhibition zone at 30 μg/disc).

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