Notes

A DNA-Damaging Oxoaporphine Alkaloid from *Piper caninum*

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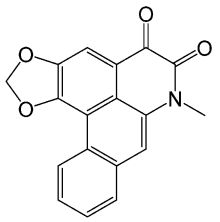
Bioassay-guided fractionation of an active organic extract of *Piper caninum*, using a sensitive yeast assay to monitor putative double-strand DNA-damaging activity, resulted in the isolation of the 4,5-dioxoaporphine alkaloid cepharadione A (**1**). Compound **1** exhibited potent inhibitory activity in a yeast cytotoxicity assay with IC_{50} values of 50.2 nM toward RS321NpRAD52 grown on glucose versus 293 nM toward the same yeast strain grown on galactose.

The genus *Piper* belongs to the Piperaceae family and encompasses over 700 species widely distributed throughout the tropical and subtropical regions of the world. Members of the *Piper* genus are of commercial, economical, and medicinal importance. Economically, the Piperaceae is employed for the production of pepper in worldwide spice markets. Plants from the genus *Piper* have been used for a number of practical applications, including as remedies in many traditional medicinal systems, such as traditional Chinese medicine, the Indian Ayurvedic system, and folklore medicines of Latin America and West Indies.[1] *P. futokadsura* has been shown to contain insect antifeedant activity.[2] *P. amalago*, distributed from Mexico to Brazil, is used for several conditions, including gastrointestinal and chest pain.[3] Phytochemical investigations of many *Piper* species have resulted in the isolation of numerous biologically active natural products including alkaloids, lignans, flavones, and chalcones.[1,4]

In yeast, DNA repair pathways include the RAD3, RAD6, and RAD52 pathways. The RAD3 pathway is associated with excision repair, the RAD6 pathway is the error-prone recombinational pathway, and RAD52 is responsible for the repair of double-strand breaks and meiotic recombination.[5] As part of our continuing effort toward the discovery of novel naturally occurring anticancer agents, a microtiter plate yeast assay has been developed for DNA-damaging agents on the basis of control of the RAD52 repair pathway. The assay employs a strain of *Saccharomyces cerevisiae* that either expresses or lacks elements of the RAD52 repair pathway under specific growth conditions, facilitating the detection of agents capable of inducing double-stranded DNA breaks. Specifically, the assay involves a strain of *S. cerevisiae*, RS321NpRAD52, which harbors a plasmid containing the RAD52 recombinant gene under the control of a GAL1 promoter. Expression of RAD52 protein in the yeast strain is thus suppressed when the strain is grown in the presence of glucose, while expression occurs on galactose-containing media.[6,7] As a control, a similar reconstructed plasmid vector lacking the RAD52 gene was introduced into the RS321N strain.[7] Using this control strain, designated RS321NYCp50, cells were assayed on a galactose medium under the same condition as the RS321NpRAD52 cells. Thus the differential growth observed in the assay could only be attributed to the difference in DNA repair capabilities rather than to the carbon source. Natural product extracts were assumed to contain DNA-damaging agents if the IC_{50} values for inhibition of RS321NpRAD52 grown on glucose and RS321NYCp50 grown on galactose were at least 3-fold smaller than the IC_{50} value for RS321NpRAD52 grown on galactose.

In the assay described above, a crude extract of *P. caninum* showed interesting activity as a putative inducer of double-strand DNA damage, exhibiting IC_{50} values of 0.05 μg/mL toward RS321NpRAD52 grown on glucose, 115 μg/mL toward RS321NpRAD52 grown on galactose, and 0.2 μg/mL toward RS321NYCp50 grown on galactose. It was thus subjected to bioassay-guided fractionation.

The crude CH_2Cl_2−CH_3OH extract of *P. caninum* was applied to a polyamide 6S column, which was washed successivley with H_2O, 1:1 MeOH−H_2O, 4:1 MeOH−CH_2Cl_2, 1:1 MeOH−CH_2Cl_2, and then 9:1 MeOH−NH_4OH. The 4:1 MeOH−CH_2Cl_2 fraction exhibited the most potent DNA-damaging activity. This fraction was applied to a C_{18} reversed-phase column, which was washed with CH_3OH−H_2O mixtures. The 4:1 CH_3OH−H_2O fraction from this column exhibited the greatest potency in the yeast assay. C_{18} reversed-phase HPLC chromatography of this active fraction led to the identification of a single active fraction having potent DNA-damaging activity. Further purification of this active fraction by C_{18} reversed-phase HPLC afforded one pure active principle (**1**).



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Compound **1** was obtained as an orange amorphous powder and displayed a molecular ion at *m*/*z* 305, indicat-

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ing that the compound contained an odd number of nitrogens. In the ^{13}C (and ^{1}H) NMR spectra, signals typical of the 4,5-dioxoaporphine skeleton were observed at δ 175.0, 156.7, 151.4, 148.3, 133.1, 132.4, 129.1 (7.91), 128.6 (9.01), 128.3, 127.5 (7.68), 126.7 (7.68), 125.7, 123.0, 121.3, 114.2 (7.54), and 109.0 (8.15).[8] Further, one methylenedioxy group [δ 103.0 (6.46)] and one methyl group [δ30.6 (3.86)] were also identified. These NMR and MS data, together with literature data, led to the identification of **1** as cepharadione A, a known oxoaporphine alkaloid.[8−10] This is the first time that an aporphine alkaloid has been isolated from *P. caninum*, even though cepharadione A and related secondary metabolites have been reported in other *Piper* species.[11]

Compound **1** inhibited the growth of the yeast strains with IC_{50} values of 50.2 nM toward RS321NpRAD52 grown on glucose, 293 nM toward RS321NpRAD52 grown on galactose, and 2.75 nM toward RS321NYCp50 grown on galactose. It is instructive to compare the potency of cepharadione A with that of streptonigrin, the compound used as the positive control in the yeast assay and having IC_{50} values of 0.283 nM toward RS321NpRAD52 grown on glucose, 7.27 nM toward RS321NpRAD52 grown on galactose, and 0.249 nM toward RS321NYCp50 grown on galactose.

Given the considerable potency of cepharadione A in the yeast strains employed here, it is notable that no biological activity has been reported for this compound previously. The activity of cepharadione A (**1**) in the RAD52 yeast assay implies that it can induce double-strand DNA damage in an intact eukaryotic cell. Thus, the cytotoxicity noted for **1** may derive from its DNA-damaging activity. Using a similar, but less sensitive agar diffusion yeast assay, the Kingston laboratory has identified several other oxoaporphine alkaloids as putative double-strand DNA-damaging agents and DNA topoisomerase inhibitors.[12]

Experimental Section

General Experimental Procedures. Polyamide 6S was purchased from Serva Electrophoresis GmbH. Silica C_{18} (40 μm) was obtained from J. T. Baker Chemicals. A Higgens Kromasil 100 C_{18} reversed-phase column (250 × 10 mm, 5 μm) was used for HPLC separations. ^{1}H NMR and ^{13}C NMR spectra were obtained on Varian Unity Inova 300 and 500 spectrometers. A low-resolution chemical ionization mass spectrum was recorded on a Finnigan MAT 4600 mass spectrometer. Yeast nitrogen base (YNB) without amino acids was obtained from Difco Laboratories. D-(+)-Glucose, D-(+)-galactose, L-histidine monohydrochloride, adenine hemisulfate salt, and streptonigrin were purchased from Sigma Chemicals. Ninety-six-well plates were obtained from Corning Inc., and a Bio-Rad Benchmark microplate reader was used to obtain the optical density (OD) of each well of yeast solution.

Plant Material. The stem of twigs of *P. caninum* were collected in Celebes, Sulawesi, on March 10, 1990. Voucher specimen Q66O8931 is preserved at the U.S. National Arboretum Herbarium, Washington, DC.

Extraction and Isolation. An organic extract of *P. caninum* was prepared by steeping the dried, ground twigs in 1:1 CH_2Cl_2−CH_3OH overnight at room temperature. The plant material was soaked in methanol. The combined organic solution was concentrated under diminished pressure to afford the organic extract. The organic crude extract of *P. caninum* showed strong DNA-damaging activity in the yeast assay (IC_{50} value: 0.05 μg/mL against RS321NpRAD52 grown on glucose, 115 μg/mL against RS321NpRAD52 grown on galactose, and 0.2 μg/mL against RS321NYCp50 grown on galactose) and was thus selected for further bioassay-guided fractionation. In a typical experiment, 1050 mg of the crude extract of *P. caninum* was applied to a 50 g polyamide 6S column; elution was carried out with H_2O, 1:1 MeOH−H_2O, 4:1 MeOH−CH_2Cl_2, 1:1 MeOH−CH_2Cl_2, and then 9:1 MeOH−NH_4OH. The 4:1 MeOH−CH_2Cl_2 fraction (483 mg) exhibited the most potent DNA-damaging activity in the yeast assay and was subjected to further fractionation on a 50 g C_{18} reversed-phase column. Elution was carried out with CH_3OH−H_2O mixtures. The 4:1 CH_3OH−H_2O fraction (26 mg) exhibited significant potency in the yeast assay. This fraction was applied to a 5 μm C_{18} reversed-phase HPLC column (250 × 10 mm), which was washed with a linear gradient of 1:4 → 4:1 CH_3CN−H_2O over a period of 60 min at a flow rate of 3.5 mL/min (UV monitoring at 280 nm). This afforded one active fraction. Using the same HPLC conditions, further purification of this active fraction resulted in the isolation of a single, pure compound (**1**), yield 1.2 mg. This compound proved to be cepharadione A. It may be noted that other less active, albeit more abundant fractions were isolated during the fractionation and may represent a source of additional DNA-damaging agents.

DNA-Damaging Yeast Assay. In the assay, each crude extract or fraction was tested at five concentrations in order to determine the IC_{50} value. Ten microliters of each extract sample was added to each of the following yeast (90 μL) cultures: RS321NpRAD52−galactose (0.67% yeast nitrogen base (YNB), 0.025 mg/mL adenine, 0.025 mg/mL histidine, 2% galactose), RS321NpRAD52−glucose (0.67% YNB, 0.025 mg/mL adenine, 0.025 mg/mL histidine, 2% glucose), and RS321NYCp50−galactose (0.67% YNB, 0.025 mg/mL adenine, 0.025 mg/mL histidine, 2% galactose). The blanks for these three solutions contained 10 μL of 10% DMSO combined with 90 μL of the proper sugar media for each strain. The negative controls consisted of 90 μL of the appropriate yeast solutions. Streptonigrin, a compound produced by *S. flocculus*,[13] is known to retard growth of cells via DNA strand scission[14] and was used as a positive control in the assay. Ten microliters of 0.1 μg/mL streptonigrin was combined with 90 μL of yeast cultures. The plates were incubated in a high-humidity incubation chamber at 30 °C for 38−42 h, and the OD of each well was measured at 595 nm. The microtiter plates were read again at 595 nm when the positive growth wells had reached an OD of 0.15−0.25. At this point, the growth inhibition was calculated by using the formula $1 - (OD_{\text{test well}} - OD_{\text{blank}})/(OD_{\text{negative control}} - OD_{\text{blank}})$.

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