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CHEMICAL CONSTITUENTS OF *ARISTOLOCHIA RIGIDA* AND MUTAGENIC ACTIVITY OF ARISTOLOCHIC ACID IV

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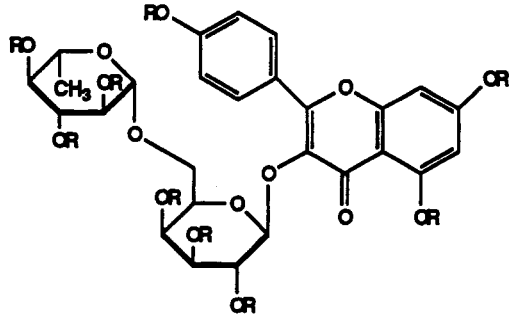
ABSTRACT.—Two aristolochic acids [**2** and **3**] and a flavonol glycoside **1** have been isolated from *Aristolochia rigida* (Aristolochiaceae). Aristolochic acid IV [**2**], the most abundant constituent, has shown a weak direct mutagenic activity in the Ames test: this action seems to be inhibited, at least in part, by metabolic reactions.

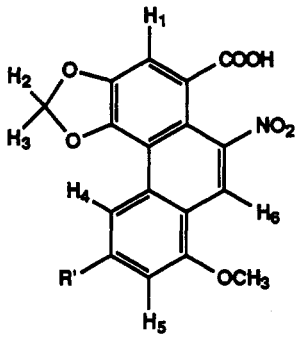
Aristolochia rigida Duch. (Aristolochiaceae) is a herbaceous plant widespread near Mogadisho (Somalia); it is carefully avoided by herbaceous animals and apparently not attacked by insects. Although this plant is not used in folk medicine in the region where it grows, we were prompted to investigate it, both because no chemical and biological studies on this plant have been reported in the literature and because plants belonging to the same genus appear to contain substances endowed with interesting biological properties (1).

The aerial parts of *A. rigida* (1.18 kg) were defatted with *n*-hexane and then extracted with Me_2CO at room temperature. The Me_2CO extract yielded a glycoside, which showed a positive Shinoda test (2) and uv absorptions at 264, 298 (sh), and 349 nm, typical of the flavonoid nucleus (3). The molecular formula $C_{27}H_{30}O_{15}$ was deduced both from elemental analysis and from fabms in positive ion mode, whose most representative peaks, besides the quasi-molecular peak at *m/z* 595 $[M+H]^+$, were at *m/z* 449 $[(M+H)-146]^+$, which suggested the loss of a deoxyhexose unit, and at *m/z* 287 $[449-162]^+$, resulting from cleavage of a hexose unit, thus showing that the aglycone unit had the formula $C_{15}H_{16}O_6$. This permitted us the deduction that the compound was a flavonoid deoxyglycosylglycoside. Indeed, acid hydrolysis afforded kaempferol, galactose, and rhamnose. More precise information about the structure of the flavonoid glycoside was obtained from its ^1H- and ^{13}C-nmr spectra (see Experimental). Comparison of the spectral data with those reported in the literature permitted identification of the compound as kaempferol-3-*O*-β-D-robinobioside [**1**] (4).

From the same extract, aristolochic acids IV [**2**] and IVa [**3**] were also ob-



1 R=H



2 R′=OMe
3 R′=OH

tained after gel filtration of the crude Me_2CO extract on Sephadex LH-20, eluting with MeOH. The ^1H- (200 MHz) and ^{13}C- (50 MHz) nmr spectra in DMSO-d_6 (see Experimental) of the two acids were in good agreement with the data reported in the literature (5).

Aristolochic acids, representative of the substituted 10-nitrophenanthrene-1-carboxylic acids, and their derivatives have been found only in this plant genus and are considered as the main active principles: they appear to have antitumor, immunomodulating, and antifertility properties (1). The mutagenicity of aristolochic acid I is reported in the literature (6), while no data are available about aristolochic acid IV, the major constituent of *A. rigida*. Therefore, we decided to submit aristolochic acid IV [**2**] to the Ames test (7). Mutagenicity tests, using the plate incorporation assay for *Salmonella typhimurium* strain TA100, were carried out. In order to simulate the metabolic activation process that takes place in vivo, the rat liver enzyme system S9 (7) was added to the plates. The mutagenic assays were carried out in triplicate, and the number of his^+ revertent colonies was scored, after incubation for 48 h.

Aristolochic acid IV increased the number of his^+ revertants up to 100 μg/plate without toxic effects. At 500 μg/plate a toxic effect took place, shown by the growth of single resistant colonies. The toxicity was more evident at 1000 μg/plate, 2500 μg/plate, and 5000 μg/plate: under these conditions, the absence of a cell layer was observed, and the toxic effects took place when the substance was assayed either with or without S9. The statistically significant dose-response curve ($r=0.924$; $p<0.01$) showed that the mutation of the TA100 strain was dependent on the amounts of aristolochic acid IV varying between 0 and 100 μg per plate (Figure 1). The assay with S9 showed no significant dose-response relationship: only variations of

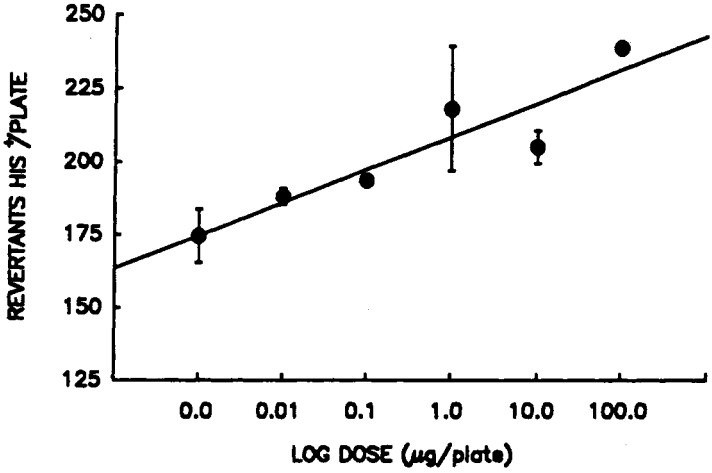


FIGURE 1. Aristolochic acid IV dose-response line in strain TA 100: $Y=2.09+1.13\chi$; $r=0.924$.

revertants per plate within spontaneous levels were noted. On the contrary, in the absence of S9, a significant increase of revertants per plate took place. These results indicated that aristolochic acid IV [**2**] is endowed with weak direct mutagenic properties, and this effect seemed to be inhibited, at least in part, by metabolic reactions.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The ir spectra were determined with PYE UNICAM spectrophotometer model SP3-300; fabms were registered in positive ion mode in thioglycerol matrix, VG ZAB instrument; ^{1}H- and ^{13}C-nmr spectra were taken on a Bruker AC 200 instrument, and chemical shifts are given in ppm from TMS as internal standard.

Lobar RP8 reversed-phase (40-63 nm Merck), Sephadex LH-20 (Pharmacia Fine Chemical), and Polyamide 6S (Riedel-de-Haen) were used for cc. Tlc was performed on RP8 reversed-phase and polyamide precoated layers (Merck).

PLANT MATERIAL.—Aerial parts of *A. rigida* were collected in June 1990, in Madina, near Mogadisho, Somalia. A voucher specimen was deposited in the Herbarium of National Somali University and another at the Dipartimento di Botanica, University of Pisa.

EXTRACTION AND ISOLATION.—Air-dried leaves and stems of *A. rigida* (1.18 kg), were defatted with hexane and then extracted with Me_2CO (1200 ml) at room temperature. The residue obtained by evaporation of the Me_2CO extract (25 g) was dissolved in MeOH, and the insoluble part (3.6 g) was identified as aristolochic acid IV [**2**]. The MeOH solution, concentrated in vacuo and shaken with H_2O, yielded a precipitate (1.83 g) which was chromatographed over Sephadex LH-20 and eluted with MeOH to give aristolochic acid IVa [**3**] (0.066 g). The aqueous filtrate was subjected to polyamide cc and eluted with MeOH-Me_2CO-PhMe (80:20:3) to give three fractions (F_1, F_2, F_3). The F_3 fraction (0.368 g) was subjected to Si gel cc and eluted with $CHCl_3$-MeOH-H_2O (6:4:1) to give kaempferol-3-*O*-β-robinobioside [**1**] (0.139 g), which was recrystallized from MeOH.

Kaempferol-3-O-β-*robinobioside* [**1**].—Yellow powder: mp 195–197°; fabms *m/z* $[M+H]^+$ 595, $[(M+H)-146]^+$ 449, $[449-162]^+$ 287; λ max (MeOH) 264, 298 sh, 349 nm; ^{1}H nmr (DMSO-d_6) δ 6.12 (1H, d, J=1.6 Hz, H-6), 6.30 (1H, d, J=1.6 Hz, H-8), 8.03 (2H, d, J=8.7 Hz, H-2′ and H-5′), 6.86 (2H, d, J=8.7 Hz, H-3′and H-6′), 5.27 (1H, d, J=7.4 Hz, H-1″), 4.41 (1H, br s, H-1‴), 1.08 (3H, d, J=6.0 Hz, H-6‴), 3.0–4.0 (10H, m, H-2″, H-3″, H-4″, H-5″, H-6″, H-2‴, H-3‴, H-4‴, H-5‴); ^{13}C nmr in agreement with published data (4).

Aristolochic acid IV [**2**].—Yellow crystals: mp 260–270° (from Me_2CO); ^{1}H nmr (DMSO-d_6) δ 7.77 (1H, s, H-1), 6.45 (2H, s, H-2 and H-3), 8.07 (1H, d, J=1.9 Hz, H-4), 6.93 (1H, d, J=1.9 Hz, H-5), 8.47 (1H, s, H-6), 4.04 (OMe), 3.95 (OMe); ^{13}C nmr in agreement with literature data (5).

Aristolochic acid IVa [**3**].—Red crystals (0.0066 g): mp 270–280° (from MeOH); ^{1}H nmr (in DMSO-d_6) δ ppm 7.85 (1H, s, H-1), 6.55 (2H, s, H-2 and H-3), 8.11 (1H, d, J=2.0 Hz, H-4), 6.88 (1H, d, J=2.0 Hz, H-5), 8.55 (1H, s, H-6), 4.06 (OMe), 8.50 (OH); ^{13}C nmr in agreement with literature data (5).

Aristolochic acid IV sodium salt.—Aristolochic acid IV (100 mg) was dissolved in 1 N NaOH (2.0 ml), and the solution was evaporated to dryness.

Ames assay.—This test is widely employed in detecting genotoxic activity of many chemical compounds (8). For the test, selected *S. typhimurium* strain TA100 was used, that spontaneously reverts from histidine dependence (auxotrophy) to histidine independence (prototrophy); this frequency of reversion (about 150 revertants per plate for TA100) is increased by the presence of base-substitution acting mutagen. To 100 ml of top agar (maintained at about 43°) were added 10 ml histidine (0.5 mM) and 10 ml biotin (0.5 mM) solutions, 0.1 ml bacteria from a fresh culture, and 0.5 ml metabolic activation mixture (when necessary). The test was replicated five times in triplicate by adding 0.1 ml of the solution of aristolochic acid IV sodium salt containing 100, 500, 1000, 2500, or 5000 μg. This mixture was mixed gently and poured on to plates containing about 25 ml of minimal-glucose agar. After the top agar had solidified, the plates were inverted and incubated at 37° for 48 h. The S9 mix (metabolic activation) was prepared from the liver of rats treated with phenobarbital. To check both TA100 strain and S9 mix, 2-acetylaminofluorene (2AAF) was used as positive control (7).

The test is positive when a statistically significant relationship is obtained between the average number of revertants per plate and the doses applied in the experimental design.

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