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3-HYDROXYURIDINE, AN ALLELOPATHIC FACTOR OF AN AFRICAN TREE, *BAILLONELLA TOXISPERMA*

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Key Word Index—*Baillonella toxisperma*; Sapotaceae; allelopathy; allelochemical; 3-hydroxyuridine; plant growth inhibitor; herbicidal activity.

Abstract—A plant growth inhibitor was isolated from *Baillonella toxisperma*, a plant which may cause an allelopathic effect in the tropical rain forest of Cameroon. From spectral and chemical evidence, the inhibitor was identified as 3-hydroxyuridine. It inhibited both hypocotyl and root growth of cucumber and radish seedlings, and also inhibited root growth of rice seedlings, but not sheath growth. The foliar treatment of 3-hydroxyuridine exhibited inhibitory effects on the growth of a wide variety of weeds; however, it was quite ineffective against the growth of the crop plant, *Zea mays*. The strong plant growth inhibitory activity, together with the occurrence in most parts of the tree at high levels, indicated that 3-hydroxyuridine may be involved in the allelopathy of the tree.

INTRODUCTION

As a part of an investigation to discover new naturally occurring bioactive substances, we conducted field research on economical plants in the tropical rain forest of Cameroon [1]. In the phytocoenological survey, *Baillonella toxisperma* (Sapotaceae) was found to be one of the most interesting trees in the forest. The tree grows in a limited area of the forest, and in the vicinity of the mature tree of *ca* 50 m height and 1.9 m diameter at breast height, only saplings of *ca* 1.6 m height are able to grow. The growing area of the saplings is very restricted under the crown (*ca* 30 m diameter) of the mature tree. Under experimental conditions, the methanolic extracts of both the aerial part and root of the tree strongly inhibited germination of cucumber (*Cucumis sativus*) and radish (*Raphanus sativus*) seed. Thus, the phenomenon was suggested to be allelopathy [2, 3] caused by specific secondary product(s) of the tree. Here we report on a possible allelopathic factor of the tree and its inhibitory activities against plant growth.

RESULTS AND DISCUSSION

Although most of the plant parts (leaf, stem and root) contained plant growth inhibitor(s), the dried stems (800 g) were selected for convenience to investigate the inhibitor. By the combination of several chromatographical techniques, the inhibitor, **1** (92 mg), was isolated as a colourless resin.

The ^{1}H NMR signals (CD_3OD, 90 MHz) at δ 8.00 (1H, *d*, $J=8$ Hz), 5.88 (1H, *d*, $J=4$ Hz), 5.80 (1H, *d*, $J=8$ Hz) and 4.0–3.5 (5H), and the UV maximum at 260 nm (log ε, 3.90) in water (pH 7.0) were very close to those of uridine [4, 5]. However **1** was positive for a ferric chloride test [6], dissimilar to uridine. The CIMS (*iso*-butane, probe) at 250 eV indicated the parent ion peak at *m/z* 261 ($[M+H]^+$, $C_9H_{12}O_7N_2+H$) and prominent fragment ion peaks at *m/z* 133 and 113, which might correspond to those derived from a sugar (ribose) and a heterocyclic (uracil) residues of uridine, respectively. Furthermore, the UV maximum at 260 nm shifted to 265 nm when measured at pH 13.5.

Hydrolysis of **1** with perchloric acid [7] gave a deribosyl compound (**2**), still positive to a ferric chloride test. The ^{1}H NMR (CD_3OD, 90 MHz) spectrum showed only two olefinic proton signals at δ 7.39 (1H, *d*, $J=8$ Hz) and 5.78 (1H, *d*, $J=8$ Hz). The high resolution EIMS (70 eV) of **2** showed a parent ion peak at *m/z* 128.0220, corresponding to a molecular formula $C_4H_4O_3N_2$ (calcd, 128.0890), suggesting that **1** has an additional oxygen atom at N^3 of uridine. Reduction of **1** with triphenylphosphine [8] gave a deoxy-derivative [EIMS (70 eV): *m/z* 245, $[M+H]^+$], which was identified as uridine by detailed spectral comparison. Thus **1** is 3-hydroxyuridine.

Treatment of **1** with acetic anhydride–pyridine gave the acetate (**3**). The 400 MHz ^{1}H NMR ($CDCl_3$) spectrum showed the signal due to H-6 as two sets of doublets at 7.42 and 7.38 ppm ($J=8$ Hz), and furthermore C-1, C-4, C-5, C-2′, C-3′, C-4′ and C-5′ were detected as doublets in the complete proton-decoupled ^{13}C NMR. The pairs of two singlets at δ 165.6 and 165.5, and 156.7 and 147.4 could be attributed to the carbonyl carbons and olefinic carbons carrying an enolic acetyl group, respectively. Thus **3** consists of two isomeric tetraacetates, whose structures were tentatively assigned as **3a** and **3b**, derived from the tautomeric *N*-oxide forms of **1**, **1a** and **1b**, respectively.

The plant growth inhibitory activity of 3-hydroxyuridine (**1**) was tested against cucumber, radish and rice seedlings. As shown in Fig. 1, 3-hydroxyuridine strongly

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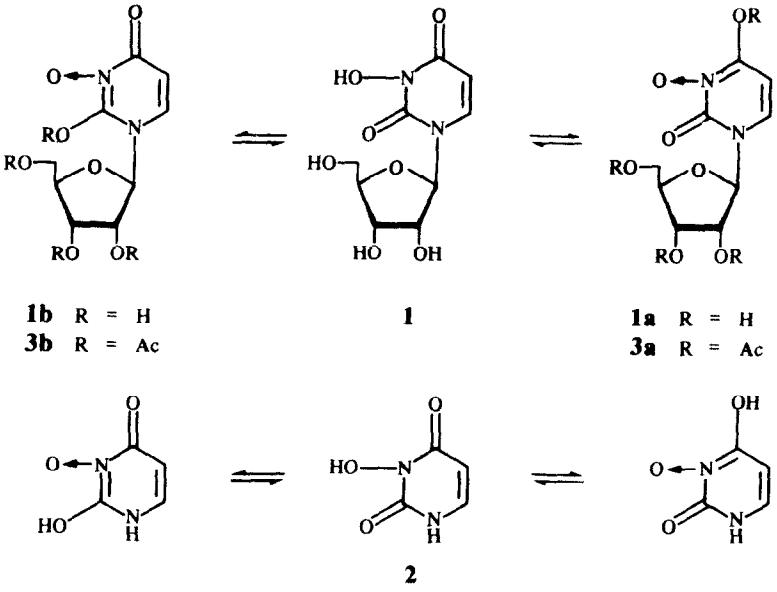

1b R = H
3b R = Ac

1

1a R = H
3a R = Ac

2

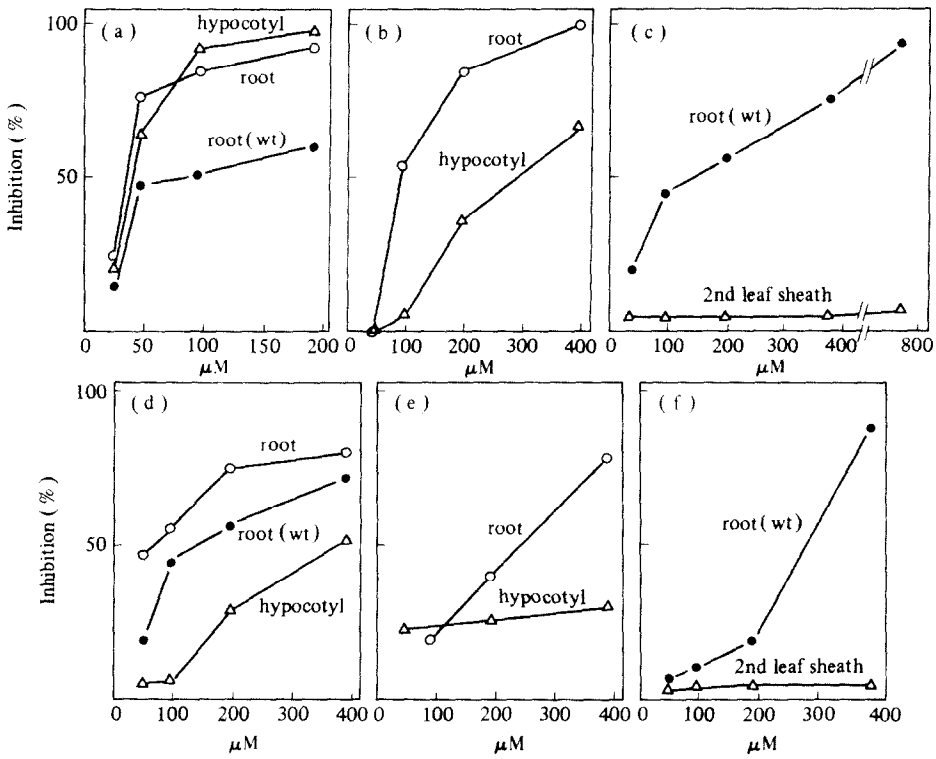

Fig. 1. Plant growth inhibitory activity. Top figures show the activity of 3-hydroxyuridine against (a): cucumber, (b): radish and (c): rice seedling, respectively. Bottom figures show the activity of 3-hydroxyuracil against (d): cucumber, (e): radish and (f): rice seedling, respectively. ○: activity against root growth evaluated by the length, ●: activity against root growth evaluated by the dry weight, and △: activity against hypocotyl growth or 2nd leaf sheath.

inhibited both the hypocotyl and root growth of cucumber, and the 50% inhibitory concentrations (I_{50}) were *ca* 40 μM. It was also inhibitory against the hypocotyl (I_{50} = 300 μM) and root growth (I_{50} = 100 μM) of radish seedlings, and against the root growth (I_{50} = 200 μM) of rice seedlings. Interestingly, **1** was quite inactive against the second leaf sheath growth of rice seedlings, even tested at 770 μM. As shown in Fig. 1d–1f, the inhibitory activities of 3-hydroxyuracil (**2**) against the root growths were basically same as **1** (I_{50} = 20, 60 and 60 μM, against cucumber, radish and rice seedlings, respectively), while the activities against hypocotyl growths of cucumber and

radish were lowered. Similarly to **1**, **2** was entirely inactive against the growth of second leaf sheath of rice seedlings. Both uridine and uracil were inactive in the test (data not shown) and therefore oxidation at N^3 was found to be important for the plant growth inhibitory activity of **1**.

For a more detailed understanding of the effect of *N*-oxidation on plant growths, oxygenation of nucleosides with *m*-chloroperbenzoic acid was conducted. Adenosine-N^1-oxide (**4**) and cytidine-N^3-oxide (**5**) were easily derived from the corresponding nucleosides as reported previously [9–11], while *N*-oxidation of uridine and thymidine was unsuccessful. The inhibitory activity (I_{50} = 250 μM) of **4** against the root growth of rice seedlings was almost equal to **1**. Cytidine-N^3-oxide (**5**) was slightly active (I_{50} = 600 μM) against the root growth of cucumber. No more remarkable activities were observed in **4** and **5**. 3-Hydroxyuridine (**1**) is, thus, the most potent plant growth inhibitor among *N*-oxygenated nucleosides.

Plant growth inhibitory activities of **1** by a foliar treatment were also tested against monocotyl weeds, *Echinochloa crus-galli* and *Setaria viridis*, and dicotyl weeds, *Pharbitis purpurea*, *Abutilon avicennae* and *Cassia tora*. As listed in Table 1, spraying an aqueous solution containing 30 mg of **1** inhibited significantly the growth of all of the weeds, while **1** was quite ineffective to *Zea mays*, an important crop plant.

GC analyses, in which **1** was detected as its tetraacetates (**3a** and **3b**), indicated that **1**, as expected from the preliminary assay, occurs not only in the stem of *B. toxisperma* but also in the leaf and root. The levels of **1** per 1 g dried leaf, stem and root were 0.60, 0.16 and 0.092 mg, respectively. From these results, it may be concluded that **1** is involved in the allelopathy of *B. toxisperma*.

Contrary to our expectations, **1** could not be detected by GC analyses in the surrounding soil. The water solubility of **1** may be unsuitable for its accumulation in the soil. However, the property should be favourable for **1** to leach from leaf and stem to move into the soil in the rain forest. 3-Hydroxyuridine (**1**) may be converted chemically or biologically into less water-soluble derivative(s) such as 3-hydroxyuracil (**2**) in the surrounding soil to cause the allelopathy.

EXPERIMENTAL

Bioassays. To a filter paper (Toyo No. 2, 5.5 cm i.d.) in a petri dish (6 cm i.d., 2 cm depth) was added a known amount of the test compound in 85% aq. MeOH. The solvent was evapd in a desiccator under red. pres. After addition of dist. H_2O (3 ml) to the dish, plant seeds (6 in cucumber, *Cucumis sativus*, 10 in radish, *Raphanus sativus*) were sowed on the paper. They were incubated at 25° under continuous light. After 96 hr, lengths of the aerial part and the root of each plant were averaged to be compared with those of a control experiment. The inhibitory activity against the growth of cucumber root was also evaluated by comparing the dried weight of the treated plant to that of a control experiment. The rice seedling test, in which the elongation of the 2nd leaf sheath and a total dry weight of the roots were measured, was conducted by the method previously reported [12, 13].

The growth inhibitory test by foliar treatment was conducted in a greenhouse run at 27° in daytime and 20° at night. *Echinochloa crus-galli*, *Setaria viridis*, *Pharbitis purpurea*, *Abutilon avicennae*, *Cassia tora* and *Zea mays* were grown by mixed planting in a vat (33 cm × 23 cm, 11 cm depth). After 10 days, an aqueous solution (5 ml) containing 30 mg (or 7.5 mg) of the inhibitor was sprayed over the whole plants, which had grown up to the 1st ~2nd leaf stage. After 20 days of this treatment, the inhibitory effect against each plant was judged by comparing the growth macroscopically to that of a control experiment. The activity was evaluated in 5 grades, as shown in Table 1.

Extraction and isolation of the inhibitor. The dried stems (800 g) of the saplings of *B. toxisperma* were extracted with MeOH. The filtrate was concentrated *in vacuo* to give an aq. extract which was partitioned with EtOAc–H_2O. The active H_2O-soluble part was chromatographed on granular charcoal eluted with H_2O of increasing amounts of MeOH. The combined active fraction, eluted with 60–100% MeOH in H_2O, was further purified by droplet counter current chromatography (DCCC) (4 mm i.d. × 40 cm length, 300 columns) with upper and lower phase of *n*-BuOH–*n*-PrOH–H_2O (2:1:3) as a stationary and a mobile phase, respectively. Final purification by preparative HPLC on ODS (YMC-PACK A-311) with water as an eluant (1 ml/min) resulted in isolation of a growth inhibitor, **1** (92 mg), as a colourless resin. It was visualized as a purplish red spot at R_f 0.20 on silica gel TLC (Kiesel gel $60F_{254}$ from Merck Co., 0.2 mm thickness) developed with an upper layer of H_2O–*n*-BuOH–AcOH (5:4:1) after spraying with a solution of 3% $FeCl_3$ in 0.5 M HCl. $[\alpha]_D^{25}$ +17.5° (MeOH; *c* 0.34), EIMS 70 eV *m/z* (rel. int.): 244 $[M-16]^+$ (0.3), 133 (21), 112 (100), CIMS (*iso*-butane, probe) 250 eV *m/z* (rel. int.): 261 $[M+H]^+$ (0.2), 245 $[M+H-16]^+$ (7), 149 (3), 133 (7), 113 (100), IR ν_{max}^{KBr} cm^{-1}: 3400, 1700, 1660.

Hydrolysis of **1** *with perchloric acid.* The inhibitor (**1**) (16 mg)

Table 1. Plant growth inhibitory activity of 3-hydroxyuridine by foliar treatment

Plant	Dose (mg)*	Activity†	Plant	Dose (mg)*	Activity†
Echinochloa	30	4	*Abutilon*	30	5
crus-galli	7.5	2	*avicennae*	7.5	3
Setaria	30	4	*Cassia*	30	5
virudis	7.5	3	*tora*	7.5	2
Pharbitis	30	5	*Zea*	30	0
purpurea	7.5	3	*mays*	7.5	0

*Thirty mg (or 7.5 mg) of 3-hydroxyuridine was dissolved in water (5 ml) and the solution was sprayed over the whole plants.

†The activity was evaluated in five grades by macroscopic examination. The activity-grade 5 means death of the plant, and the grade 0 means no inhibition. The grades 4, 3 and 2 indicate to be strongly active, active and weakly active, respectively.

and 70% $HClO_4$ (250 μl) in a sealed tube were heated at 100° for 1 hr. The reaction mixture was neutralized with NH_4OH, concd, and purified by CC on granular charcoal. The column was first eluted with excess of water (5 × bed vol.) to eliminate the salt and then with water of increasing amounts of MeOH. 3-Hydroxyuracil (**2**) (7 mg) was obtained in a fraction eluted with 60–80% MeOH in water. UV λ_{max}^{MeOH} nm (log ε): 256 (3.94), ^{1}H NMR (90 MHz, $CDCl_3$): δ 5.78 (1H, *d*, *J* = 8 Hz), 7.39 (1H, *d*, *J* = 8 Hz), IR ν_{max}^{KBr} cm^{-1}: 3100, 2900, 1700, 1670, 1630.

Reduction of **1** *with triphenylphosphine.* The inhibitor (**1**) (7 mg) and triphenylphosphine (8.5 mg) was refluxed in ethyleneglycol (0.5 ml) for 40 min. H_2O was added to the mixture which was passed on a column of granular charcoal (1 g). The column was first eluted with excess of H_2O (5 × bed vol.) to eliminate ethyleneglycol and then 70% aq. MeOH (5 × bed vol.) to give uridine (4 mg).

Acetylation of **1**. The inhibitor (**1**) (12 mg) was acetylated with pyridine and Ac_2O. Usual work-up and purification by prep. TLC on silica gel (20 × 20 cm) developed with C_6H_6–EtOAc (2:1) gave a mixture (11 mg) of two tetraacetates, **3a** and **3b**. IR ν_{max}^{KBr} cm^{-1}: 1800, 1750, 1700, 1620, EIMS 70 eV *m/z* (rel. int.): 429 $[M+H]^+$ (0.2), 259 (66), 171 (3), 157 (20), 139 (100), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42 and 7.38 (total 1H, each *d*, *J* = 8 Hz), 5.99 (1H, *d*, *J* = 5 Hz), 5.88 (1H, *d*, *J* = 8 Hz), 5.35 (2H, *m*), 4.36 (3H, *m*), 2.37 (3H, *s*), 2.15 (3H, *s*), 2.10 (3H, *s*), ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.5, 167.0, 168.9, 165.6 and 165.5 (C-2 and C-4 carbonyl carbon), 156.7 and 147.4 (C-4 and C-2 enolic carbon), 137.1 and 137.0 (C-6), 102.2 and 102.0 (C-5′), 88.4 (C-1′), 80.2 and 80.0 (C-4′), 72.9 and 72.8 (C-3′), 70.1 and 69.9 (C-2′), 63.0 and 62.9 (C-5′), 20.9, 20.6, 20.5, 17.8.

N-Oxidation of adenosine and cytidine [11]. To adenosine (100 mg) in 30% aq. dioxane (10 ml) was added *m*-chloroperbenzoic acid (129 mg) and the reaction mixture allowed to stand for 12 hr. Pd/C was added to the mixture until excess of *m*-chloroperbenzoic acid was degraded. After filtration and evapn, the residue was purified by prep. TLC with an upper layer of *n*-BuOH–AcOH–water (4:1:5) followed by CC on granular charcoal eluted first with an excess of H_2O and then 70% MeOH in H_2O. The fraction eluted with 70% MeOH in H_2O gave adenosine-N^1-oxide (**4**) (68.4 mg). $[\alpha]_D^{21}$ −40.0° (H_2O; *c* 0.80), EIMS 70 eV *m/z* (rel. int.): 247 $[M-18-18]^+$ (0.2), ^{1}H NMR (90 MHz, D_2O): δ 9.03 (1H, *s*), 8.87 (1H, *s*), 6.51 (1H, *d*, *J* = 4 Hz), 4.8–4.0 (5H, *m*), UV $\lambda_{max}^{H_2O}$ nm (log ε): 237 (4.56), 254 (3.85), IR ν_{max}^{KBr} cm^{-1}: 3300, 1670. Cytidine-N^3-oxide (**5**) (66 mg) was obtained from cytidine (100 mg) in a similar manner. $[\alpha]_D^{21}$ +32.3° (H_2O; *c* 0.53), EIMS 70 eV *m/z* (rel. int.): 259 $[M]^+$ (0.2), ^{1}H NMR (90 MHz, D_2O): δ 8.33 (1H, *d*, *J* = 8 Hz), 6.76 (1H, *d*, *J* = 8 Hz), 6.34 (1H, *d*, *J* = 4 Hz), 4.8 ~ 4.0 (5H, *m*), UV $\lambda_{max}^{H_2O}$ nm (log ε): 228 (4.26), 273 (2.44), IR ν_{max}^{KBr} cm^{-1}: 3300, 1650.

GC analysis. Each dried plant part (10 ~ 50 g) was extracted with MeOH for 2 weeks. A portion of the aq. part of the MeOH extract was once purified on granular charcoal (1 g) eluted with H_2O (60 ml), 40% aq. MeOH (60 ml) and 70% aq. MeOH (300 ml), successively. One-tenth of the fraction eluted with 70% aq. MeOH was acetylated in a usual manner. The acetylated mixture was filled up with EtOAc by 100 μl, and a portion (2 μl) of the solution was injected into GC with an FID: FFS capillary column OV-1 (50 m × 0.24 i.d.), column temp. 235°, inj. and detector temp. 250°, N_2 flow rate 50 ml/min. The tetraacetates were detected as a single peak at R_t 6.9.

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