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L-2-OXALYLAMINO-3-AMINOPROPIONIC ACID, AN ISOMER OF *LATHYRUS SATIVUS* NEUROTOXIN

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Key Word Index—*Lathyrus sativus*; Leguminosae; non-protein amino acid; L-2-oxalylamino-3-aminopropionic acid.

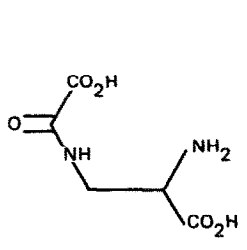
Abstract—The first chemical synthesis of L-2-oxalylamino-3-aminopropionic acid, an isomer of the *Lathyrus sativus* neurotoxin, is described. Studies on its biological properties are reported. Experiments with L-3-[^{14}C-oxalyl]amino-2-aminopropionic acid show that the amount of 2-oxalylamino isomer detectable in seed extracts can be accounted for by rearrangement which occurs during isolation.

INTRODUCTION

The human disease neurolathyrism occurs after prolonged ingestion of the seeds of *Lathyrus sativus*, a legume whose neurotoxic component is the non-protein amino acid L-2-amino-3-oxalylaminopropionic acid (**1**) [1]. A common contaminant of toxin preparations isolated from seed [2,3] or obtained by chemical synthesis [1,4] is 2-oxalylamino-3-aminopropionic acid (**2**). This compound is of interest because its toxicity is unknown and its origin in plant extracts is uncertain.

The 2-oxalylamino isomer (**2**) is detectable by paper electrophoresis of L-3-oxalylamino-2-aminopropionic acid (**1**) preparations as a second, slower moving band which stains grayish-purple with ninhydrin [2,3]. Although it can be removed from crystalline preparations of **1** by repeated washing of the crystals with cold water, solutions of **1** rearrange on standing to form an equilibrium mixture with **2** [2]. The rearrangement product forms oxalic acid and 2,3-diaminopropionic acid on hydrolysis and lacks a free 2-amino group, as indicated by the failure of pre-treatment with cupric nitrate to affect color development with ninhydrin [2].

Whether the 2-oxalylamino isomer (**2**) is a natural component of the plant or is formed during isolation procedures is unclear. Bell and O'Donovan [2] specifically rejected the idea that **2** might be an artifact, despite their observations that it can be formed by rearrangement *in vitro*. Using isolation methods intended to minimize rearrangement (50% EtOH in the cold for 4 hr), they recovered **2** as about 5% of the total toxin from seed extracts of *L. sativus*, *L. cicera* and *L. clymenum*. With another isolation method which involved the use of ion exchange resins, **2** comprised 50% of the total toxin recovered from *L. latifolius* seed. Similar conclusions regarding the natural occurrence of **2** were reported by Roy and Rao [3] who investigated the distribution of the isomer in freeze-dried 30% EtOH extracts of 12 *L. sativus* strains. They reported that **2** constituted a relatively constant fraction (3.8–7.9%) of the total toxin.



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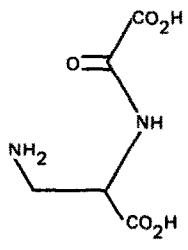

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Doubts regarding the natural origin of **2** arise, however, when the specificity of the enzyme which attaches oxalyl CoA to L-2,3-diamino-propionic acid is considered. This enzyme forms the 3-oxalylamino isomer [5]. If the 2-oxalylamino isomer cannot be formed in the plant by any known enzymic pathway but *can* be formed by rearrangement, then the possibility that the 2-oxalylamino isomer is an artifact of isolation deserves attention.

Studies on the toxicity of 2-oxalylamino-3-aminopropionic acid (**2**) were undertaken in our laboratory as part of our work on the mechanism of action of L-3-oxalylamino-2-aminopropionic acid (**1**). The 2-oxalylamino isomer (**2**) was synthesized, and its biological properties were investigated. Quantitative studies dealing with the rearrangement of L-3-[^{14}C-oxalyl]amino-2-aminopropionic acid (**1**-^{14}C) during procedures used routinely in isolation of **1** from seed were carried out. These show that the quantities of **2** reported to occur naturally can be accounted for by the rearrangement which occurs during isolation.

RESULTS AND DISCUSSION

Synthesis of L-2-*oxalylamino*-3-*aminopropionic acid*

The compound was prepared by condensation of L-3-benzyloxycarbonylamino-2-aminopropionic acid [6] and oxalyl chloride followed by hydrogenolysis of the 3-amino protecting group (see Experimental). The crude product contained predominantly L-2-oxalylamino-3-

aminopropionic acid, but some L-3-oxalylamino-2-aminopropionic acid was also present, as indicated by paper electrophoresis [3]. Pure **2** was isolated after repeated washing of the crude product with Me_2CO and H_2O. The purified product was identical with the compound detected in *L. sativus* seeds [1] in electrophoretic mobility [3], color reactions [2,7] and in GLC R_t of the *N*-dimethylaminomethylene methyl ester derivatives [8].

Neurotoxicity of the 2-oxalylamino isomer

Immature animals develop neurological symptoms rapidly after injection of 3-oxalylamino-2-aminopropionic acid (**1**) [1,9]. The most widely used biological assay depends upon the characteristic response of a 1-day-old chick [1,10,11]. An injection of 300 μg/g body wt produces somnolence, flaccid paralysis of the neck muscles and collapse within about 30 min. It was, therefore, of interest to test the 2-oxalylamino isomer (**2**) in this system. One-day-old chicks were injected with either 300 or 600 μg/g body wt of **2**, 300 μg 3-oxalylamino isomer (**1**) or physiological saline solutions whose Na content was equal to that of the neutralized oxalyldiaminopropionic acid solutions. Neither the 2-oxalylamino isomer (**2**) nor saline solutions had effect at either dosage level. Injection of the 3-oxalylamino isomer (**1**) produced the usual neurological symptoms within 30 min.

The experiment was repeated, using 3-day-old C57BL/6J mice as subjects. The LD_{50} for **1** in such animals is 300 μg/g body wt; 500 μg/g body wt invariably is fatal [12]. When 500 μg/g body wt of **1** was injected into 3-day-old mice, animals assumed a characteristic hunched posture, suffered severe muscle spasms, gasped for breath and died within 30 min. An equivalent dose of **2** produced no symptoms and no deaths.

To test the possibility that **2** might potentiate the effects of **1**, 3-day-old mice were injected with a mixture of the two isomers (300 μg/g body wt of each). The severity and time course of the symptoms were not noticeably different in animals injected with a mixture of the two isomers than in mice injected with 300 μg/g of the 3-oxalylamino isomer (**1**) alone.

Glutamate antagonism

An interesting property of 3-oxalylamino-2-aminopropionic acid (**1**) is that it behaves as a potent competitive inhibitor of L-glutamic acid transport into resting yeast cells [4]. A 1:1 molar ratio of **1** to L-glutamate depressed glutamate[^{14}C] transport into resting yeast cells by 88% after 20 min. Tested in this system at the same concentration, **2** had no effect. Potentiation of **1** by **2** in this system does not occur as indicated by the following experiment. When an equimolar mixture of **1**:**2**:glutamate-[^{14}C] was tested, inhibition of glutamate[^{14}C] transport was no greater than in the experiment with only **1** as antagonist.

A second characteristic of the 3-oxalylamino isomer (**1**) is that it inhibits yeast growth when added to a chemically defined medium [4]: 0.2 μg/ml depresses growth to 50% of control values. When **2** was tested in this assay, there was no effect on growth.

Formation of 2-oxalylamino-3-aminopropionic acid during isolation procedures

Others investigators [2,3] have suggested that the 2-oxalylamino isomer (**2**) occurs naturally in plants, since they found it in small amounts (about 5% of total toxin) in plant extracts prepared in the cold and analyzed promptly to rule out the possibility of rearrangement *in vitro*. Using 3-[^{14}C-oxalyl]amino-2-aminopropionic acid (**1**-^{14}C), we tested the possibility that the small amount of **2** found in plant extracts might form during electrophoresis. This proved to be the case. When 30000 dpm of **1**-^{14}C, which had been purified by paper electrophoresis, was subjected to electrophoresis as described by Roy and Rao [3], 94.6% of **1**-^{14}C was recovered unchanged; 5.4% was recovered as the 2-oxalylamino isomer (**2**-^{14}C). A similar experiment with 2-(^{14}C-oxalyl)-amino-3-aminopropionic acid (**2**-^{14}C), which had been purified by paper electrophoresis, demonstrated that this compound also rearranges during electrophoresis: 78.9% was recovered unchanged, 19.2% as the 3-oxalylamino isomer (**1**-^{14}C).

Isolates of 3-oxalylamino-2-aminopropionic acid (**1**) from *L. sativus* seeds frequently contain *ca* 30% of **2**. It was therefore of interest to determine to what extent **2** is formed during isolation procedures routinely used in our laboratory. We therefore carried 90000 dpm of purified 3-[^{14}C-oxalyl]amino-2-aminopropionic acid (**1**-^{14}C) through the isolation procedure described by Rao *et al.* [1]. About 75% of the radioactivity was recovered unchanged; 25% of the radioactivity was recovered as the 2-oxalylamino isomer (**2**-^{14}C). Most of the rearrangement occurred during chromatography on Dowex 50 (H^+) and when electrophoretically homogenous **1**-^{14}C was carried through this procedure, 17% was recovered after electrophoresis as **2**-^{14}C.

In summary, experiments described here show that L-2-oxalylamino-3-aminopropionic acid (**2**) has neither the neurotoxic nor the glutamate-antagonizing properties characteristic of the *L. sativus* neurotoxin, L-3-oxalylamino-2-aminopropionic acid (**1**). They show that the quantities of **2** detected in seed extracts approximate those formed by rearrangement during isolation and electrophoresis. Our experiments do not rule out the possibility that some rearrangement of **1** to **2** may occur within the plant. However, it appears that any *in vivo* rearrangement is of minor quantitative importance. If any 2-oxalylamino isomer is naturally present in the plant, then one must assume that it formed either by a presently unknown enzymic reaction or by non-enzymic rearrangement. In the latter case, the presence of **2** in the plant may be considered an artifact in that it does not represent a purposeful biological transformation.

EXPERIMENTAL

Preparation of L-*3-amino-2-oxalylaminopropionic acid*

L-*3-Benzyloxycarbonylamino-2-aminopropionic acid* was prepared from L-2,3-diaminopropionic acid (Calbiochem, La Jolla, CA) by the method of ref [6].

L-*3-Benzyloxycarbonlyamino-2-oxalylaminopropionic acid.* To a chilled soln of oxalyl chloride (32 ml) in 300 ml of dry dioxane was added 7.71 g (32.4 mmol) L-2-amino-3-benzyloxycarbonylaminopropionic acid. The mixture was stirred at room temp. for 2.5 hr, and was then poured into 200 ml crushed ice. The soln was made basic by the addition of solid $NaHCO_3$ and extracted with EtOAc. The organic layer was discarded. The aq layer was acidified with conc HCl, and extracted with EtOAc (×3). Combined organic extracts were dried ($MgSO_4$), and solvent removed to afford 6.39 g (63%) of the product, a viscous oil which was pure enough for preparative purposes. A pure sample was obtained by dissolving

a sample of the crude material in H_2O, treatment with charcoal, and lyophilization. Drying *in vacuo* (65°) gave the pure material as the hemihydrate, a pale yellow oil, $[\alpha]_D^{22.5}$ + 45.7° (c = 0.86, H_2O); PMR 60 MHz (acetone-d_6) 9.4 (*s*, 2H), 8.5 (broad *d*, *J* 9 Hz, 1H), 7.33 (*s*, 5H), 6.7 (broad *t*, *J* 5 Hz, 1H) 5.10 (*s*, 2H), 4.67 (*m*, 1H), and 3.78 (*m*, 2H); IR ($CHCl_3$) 3400, 3050, 1745, 1510, and 1200 cm^{-1}. (Found: C, 48.94; H, 4.69; N, 8.62. $C_{13}H_{14}N_2O_7 \cdot \frac{1}{2}H_2O$ requires: C, 48.90; H, 4.74; N, 8.78).

L-3-*Amino-2-oxalylaminopropionic acid.* L-3-Benzyloxycarbonylamino-2-oxalylaminopropionic acid (2.23 g, 7.2 mmol) was dissolved in 100 ml MeOH, and the soln diluted with 100 ml 10% aq. HOAc. The soln was treated with 1 g 10% Pd on C, and the mixture hydrogenated at room temp and normal pressure for 3 hr. The catalyst was removed, and the MeOH removed under red. pres. The aq residue was freeze-dried and the product suspended in 10 ml H_2O, collected and washed with Me_2CO tone to give 1.06 g (76%) of crude material. Repetition of the H_2O and Me_2CO washes gave the pure product as the hemihydrate, mp 170–172° (gas evolution); $[\alpha]_D^{21}$ + 14.7° (c = 2.00, 1.0 *N* KOH); IR (Nujol) 3600, 3500, 3450, 3300, 3050, 1700, 1610, 1500, 1290, and 1235 cm^{-1}. (Found: C, 32.65; H, 4.87; N, 15.23. $C_5H_8N_2O_5 \cdot \frac{1}{2}H_2O$ requires: C, 32.43; H, 4.86; N, 15.14%). Paper electrophoresis [3] of the product (pH 2.2, 300 V, 4 hr) revealed a single ninhydrin-positive compound. Its electrophoretic mobility and color reactions were identical to those of the presumed 2-oxalylamino component of a mixture of oxalyldiaminopropionic acid isomers isolated from *L. sativus* seeds. (The natural product contained *ca* 65% of **1** and 35% of **2**, as indicated by intensity of ninhydrin color after separation in electrophoresis.) The *N*-dimethylaminomethylene methyl ester [8] of the synthetic material had the same GLC retention temp (237°) as that of one component of the natural product. (Thermal conductivity detector, He, 40 ml/min; temp programmed 117°–300° at 4°/min; injector 247°; detector 243°; 3 m × 3 mm glass; 3% OV-17, injected on column.) The second component of the natural product was identical in retention temp (242) to the *N*-dimethylaminomethylene methyl ester derivative [8] prepared from authentic (**1**) [4].

L-3-[^{14}*C-oxalyl*]*amino-2-aminopropionic acid* (1-^{14}*C*). Unlabelled oxalic acid (10 mg) was combined with 26 μCi of oxalic acid[^{14}C] dihydrate (74 mCi/mmol) purchased from Amersham-Searle. The mixture was converted to dimethyl oxalate [13] and 0.04 mmol was reacted with L-2,3-diaminopropionic acid (0.07 mmol) as described by Rao *et al.* [1], except that the coupling step was carried out at pH 7. Paper electrophoresis [3] of the product (pH 2.2, 300V, 4 hr) revealed two ninhydrin-positive bands. The faster moving component traveled 4.05 cm toward the anode and was identical in color reactions and electrophoretic mobility to authentic **1**. The slower moving band travelled 2.50 cm toward the anode and exhibited the grayish–purple color characteristic of **2**. The isomeric oxalyldiaminopropionic acids were recovered by elution from replicate paper strips. Recovery of the radioactivity from one such strip was quantitative, 69.5% was recovered as **1**-^{14}C and 30.0% as **2**-^{14}C. The purified product had a sp act of 240 μCi/mmol and was equal in biological activity to authentic **1** when tested in a yeast growth assay [4]. (Low sp act **1** was synthesized primarily to determine the radiochemical purity of the oxalate[^{14}C].) *Animal experiments* were carried out with 1-day-old female chicks from the University of Illinois colony of cross-breed broilers (New Hampshire males × Columbian females) or with 3-day-old mice reared in our laboratory from C57B1/6J parents. Solns of **1** and **2** were adjusted to pH 7.5 with NaOH and administered by intraperitoneal injection. *Yeast experiments* were carried out as described in ref. [4] using strain αS-288C-27.

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