THE IDENTIFICATION OF AN ACTIVE METABOLITE OF TREMORINE

Arthur K. Cho, Wilford L. Haslett and Donald J. Jenden

Department of Pharmacology
UCLA Medical Center
Los Angeles 24, California

Received June 2, 1961

Tremorine (1, 4-bis-(pyrrolidino)-butyne-2) is a compound which induces tremor and spasticity in several species of laboratory animals (Everett, 1956). These effects are antagonized by agents useful in Parkinson's disease, and as a result there is considerable interest in tremorine as a pharmacological tool. Kocsis (1960) has reported the conversion of tremorine to an active metabolite which is responsible for the pharmacological effects. This communication reports the isolation, identification and synthesis of an active metabolite of tremorine from incubation mixtures containing minced rabbit liver.

The active metabolite was detected and assayed pharmacologically by intravenous injection into mice. Whereas tremorine causes its characteristic effects only after a latent period of a few minutes, its active metabolite induces violent generalized tremor, spasticity, hypokinesia, and parasympathomimetic effects immediately after injection by the intravenous route. Threshold doses cause slight spasticity, with tremor appearing only on restraint. For purposes of rough measurement, a unit was defined as the dose per Kg. which on intravenous injection in mice gave this threshold effect. It proved to be equal to approximately 15 μg. of 2-oxotremorine base. Ascending paper chromatography was also used to identify the active metabolite in extracts; a characteristic pink spot was obtained with ninhydrin, with an R_f of 0.60 after development in n-butanol-acetic acid-water (4:1:5) on Whatman 3 MM paper.

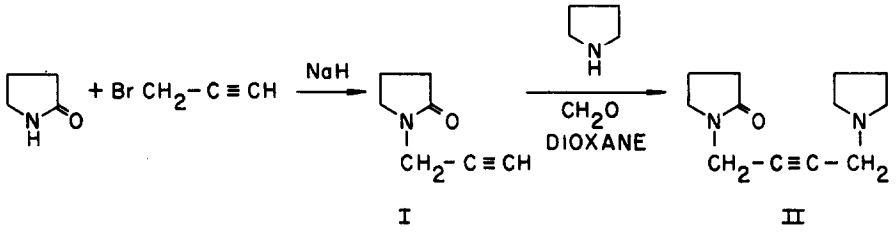
Fresh liver was obtained from male, albino rabbits weighing 2 - 3 Kg. Each animal was killed by a blow at the base of the skull and was rapidly exsanguinated by severing the great vessels of the neck. The liver was immediately excised and the gall bladder discarded. Two or three livers (about 200 gms. total) were minced and added to 2 liters of Krebs bicarbonate solution at 38°C, vigorously stirred and oxygenated with 95% O_2 and 5% CO_2 supplied through a fritted glass bubbler. Tremorine base was dissolved in a small amount of water, adjusted to pH 7.5 with hydrochloric acid and added to the incubation mixture to make a final concentration of 0.026 M. The pH of the mixture was maintained at 7.5 by occasional adjustments throughout the incubation period. Maximum yield of the metabolite was obtained within five or six hours, at the end of which time the incubation was stopped and the mixture adjusted to pH 2.0 with concentrated hydrochloric acid.

The crude incubate (usually 2 liters total volume) at pH 2.0 was heated at 100° for 2 - 3 minutes. It was cooled rapidly, stirred for 15 minutes at room temperature with 10 gm. activated bone charcoal, and filtered through a Celite pad. The resulting colorless to pale green filtrate was brought to pH 5.0 with sodium hydroxide and finally to pH 10 with potassium carbonate. The solution was extracted 3 times with 200 ml. aliquots of chloroform and the aqueous layer was discarded. This chloroform layer was extracted 3 times with 60 ml. aliquots of 1.0 M citric acid. The aqueous phase was then adjusted to pH 5.5 and extracted 6 times with equal volumes of chloroform.

The combined chloroform extracts obtained above were concentrated at reduced pressure to about 25 ml. Ethanol was added and distilled so that a solution of the metabolite in 25 ml. of ethanol was obtained. This solution was diluted with an equal volume of water and passed through a column of Bio-Rad AG50 X 4 ion exchange resin in hydrogen form. Gradient elution between 1 and 3 N hydrochloric acid gave the metabolite essentially free of tremorine as assayed by paper chromatography. The fractions containing the metabolite were combined, brought to pH 10 as above, and extracted with chloroform. The chloroform solution was concentrated and again ethanol was

added and distilled to give about 25 ml. of ethanol solution. This solution was titrated and a slight excess (10%) of picrolonic acid in hot methanol was added. Cooling gave crystals of m.p. 158-159° after recrystallization from acetone. Analysis and neutral equivalent indicated only one of the nitrogens of the metabolite was basic and that 1 gm. atom of oxygen had been added.

Anal. Calcd. for $C_{22}H_{26}N_6O_6$: C, 56.17; H, 5.57; N, 17.86. Found: C, 56.04; H, 5.43; N, 17.57.



I II

A likely structure appeared to be 1-(2-oxopyrrolidino)-4-pyrrolidino-butyne-2 (II). This compound was prepared by a Mannich reaction between 1-propargyl-2-pyrrolidone (I), pyrrolidine, and paraformaldehyde.

N-Propargyl-2-Pyrrolidone (I).

A solution of 15 gm. (0.176 moles) of pyrrolidone in 50 ml. of dry toluene was added dropwise to a stirred suspension of 9 gm. (0.2 moles) of sodium hydride (53% suspension in mineral oil) in 100 ml. dry toluene. The reaction mixture was then heated and refluxed for 2 hours and cooled. Next, a solution of 24 gm. (0.2 moles) of propargyl bromide in 50 ml. of dry toluene was added over a period of 2 hours, after which the mixture was again heated for 1 hour, cooled and allowed to stir overnight at room temperature. Filtration through celite followed by evaporation of the filtrate gave a dark oil which was placed on an alumina column. Elution with hexane, then hexane-ether (1:1), and evaporation of the combined hexane-ether fractions gave 5 gm. of a yellow oil which darkened on stand-

ing. This material was used in the following experiment without further purification.

1-(2-oxopyrrolidino)-4-Pyrrolidino-butyne-2 (II).

A solution of 2.4 gm. (20 mmoles) of the oil obtained above, 1.9 ml. pyrrolidine (22 mmoles) and 0.72 gm. paraformaldehyde (24 mmoles) in 5 ml. of dioxane was heated at 100° for 10 hours. The resulting dark solution was cooled and added to 50 ml. of water. The aqueous solution obtained was made acid with dilute hydrochloric acid and extracted 3 times with ether. The aqueous residue was made basic with sodium hydroxide and extracted 4 times with chloroform. Evaporation of the chloroform and distillation under reduced pressure gave, as the main fraction, 1 gm. of a pale yellow oil, boiling at 150-155°/0.6mm; infrared, 1685 cm^{-1}. A portion of this distillate was converted to its picrolonate salt which melted at 157-159° after 2 recrystallizations from acetone. The infrared spectrum and mixed melting point of the biogenetic and synthetic amine picrolonates indicated the two compounds were identical. The pharmacological activities of the two compounds were also indistinguishable.

Anal. Calcd. for $C_{22}H_{26}N_6O_6$: C, 56.17; H, 5.57; N, 17.86. Found: C, 56.51; H, 5.59; N, 17.82.

References:

Everett, G.M., Blockus, L.E. and Shepperd, I.M., Science, 124: 79 (1956).
Kocsis, J.J. and Welch, Richard M., The Pharmacologist, 2: 87 (1960).

This work has been supported by the U.S. Public Health Service Grant B-3007.