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Serotonergic activity of a novel tetrahydro-β-carboline

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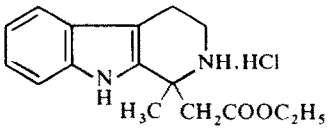
There is much pharmacological interest in tetrahydro-β-carbolines due to their possible endogenous synthesis in brain from serotonin [1–6]. Neurochemical studies *in vitro* indicated that 1,2,3,4-tetrahydro-β-carboline and 6-methoxy-1,-2,3,4-tetrahydro-β-carboline elevated serotonin levels in the brain of mice and rats [7, 8]. Studies *in vitro* indicated that tetrahydro-β-carboline analogs inhibited the metabolism of serotonin by monoamine oxidase (MAO) [9–11] or the neuronal uptake of serotonin [12–14] or increased serotonin syntheses [15]. Here we report the synthesis and activity of (±)1-ethoxycarbonylmethyl-1-methyl-,1,2,3,4-tetrahydro-β-carbolin-2-ium chloride (carbonylmethyl-THBC). This novel tetrahydro-β-carboline increases brain serotonin levels in mice but has little effect on MAO activity. However, carbonylmethyl-THBC does increase the neuronal release of serotonin *in vitro* and has agonist activity at serotonergic sites on the isolated rat fundus preparation.



Carbonylmethyl-THBC

In all studies, male albino mice (20–25 g) and rats (180–200 g) of the Fullinsdorf strain were used. Carbonylmethyl-THBC was dissolved in aqueous-Tween 80 (1%, v/v) for administration to animals. Control animals received an equivalent volume of the aqueous-Tween 80 vehicle. For all neurochemical studies, *in vitro* carbonylmethyl-THBC was dissolved in distilled water.

MAO activity *in vitro* was determined by the method of Jarrott [16] using a 10 vol. mouse brain homogenate in distilled water as the enzyme source. Substrates studied were 5-hydroxy[side chain 2-^{14}C]tryptamine creatinine sulfate ([^{14}C]5-HT, 56 mCi/m-mole, Amersham) and [side chain 2-^{14}C]tyramine hydrochloride ([^{14}C]tyramine, 55 mCi/m-mole, Amersham) over the concentration range 10^{-4} M to 2×10^{-5} M for [^{14}C]5-HT and 2.5×10^{-3} M to 5×10^{-4} M for [^{14}C]tyramine. Carbonylmethyl-THBC and standard substances were preincubated with the enzyme preparation for 5 min prior to addition of substrate.

The neuronal uptake of 10^{-8} M 1-[7-^{3}H]noradrenaline hydrochloride ([^{3}H]NA, 9.1 Ci/m-mole, Amersham) and 5×10^{-8} M 5-hydroxy[G-^{3}H]tryptamine creatinine sulfate ([^{3}H]5-HT, 11.1 Ci/m-mole, Amersham) was measured using a crude synaptosomal (P_2) preparation of mouse or rat brain cerebral cortex. Carbonylmethyl-THBC and standard substances were preincubated with synaptosomes for 5 min at 37° before addition of substrate [17].

The neuronal release of serotonin was studied using a modification of the method of Pylatuk and McNeil [18]. A crude synaptosomal (P_2) preparation from mouse or rat brain cortex was incubated for 15 min at 37° with 10^{-7} M [^{3}H]5-HT (11.1 Ci/m-mole). The synaptosomes were washed twice in cold Krebs phosphate buffer (pH 7.4) to achieve a constant efflux of [^{3}H]5-HT. Carbonylmethyl-THBC was then added to aliquots of washed synaptosomes. Release was studied over a 2-min period of 37° followed by centrifugation at 12,000 *g* for 10 min. Aliquots of the supernatant fluid were sampled and the synaptosomal pellets were dissolved in ethanol for liquid scintillation counting. Results were expressed as a percentage of [^{3}H]5-HT released compared to that retained in the synaptosomal pellet.

Carbonylmethyl-THBC was compared with serotonin for agonist activity on the isolated fundus strip prepared from the stomach of the rat. Contractions of the fundus were recorded against a 2 g weight using a Gould isotonic transducer.

Noradrenaline (NA) and dopamine (DA) concentrations were assayed by the trihydroxyindole fluorimetric method [19] after separation on a Dowex 50-W cation exchange column [20]. The endogenous concentrations of 5-hydroxytryptamine (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) in mouse brain were determined by measuring native

Table 1. Effect of carbonylmethyl-THBC on mouse brain serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), noradrenaline (NA) and dopamine (DA) concentration *

		5-HT (μg/g)	5-HIAA (μg/g)	NA (μg/g)	DA (μg/g)
Control		0.35 ± 0.02	0.67 ± 0.02	0.26 ± 0.02	0.25 ± 0.04
Carbonylmethyl-THBC	3 mg/kg	0.33 ± 0.01	0.59 ± 0.05†		
	10 mg/kg	0.43 ± 0.01‡	0.70 ± 0.03		
	30 mg/kg	0.56 ± 0.01‡	0.67 ± 0.03	0.25 ± 0.01	0.28 ± 0.02
Harmaline	1 mg/kg	0.45 ± 0.01‡	0.53 ± 0.01§	0.29 ± 0.04	0.19 ± 0.02
	3 mg/kg	0.48 ± 0.02‡	0.54 ± 0.01§	0.31 ± 0.02†	0.23 ± 0.03
	10 mg/kg	0.51 ± 0.01‡	0.51 ± 0.01‡	0.23 ± 0.01	0.24 ± 0.02

* Each value is the mean ± S.E.M. from six animals that received carbonylmethyl-THBC, harmaline or vehicle intraperitoneally 30 min previously.

† Significance of difference between means of treated and control groups (Student's *t*-test), $P < 0.05$.

‡ Significance of difference between means of treated and control groups (Student's *t*-test), $P < 0.001$.

§ Significance of difference between means of treated and control groups (Student's *t*-test), $P < 0.01$.

fluorescence after solvent separation [21, 22]. The native fluorescence of carbonylmethyl-THBC occurs at similar wavelengths (excitation 295 nm, emission 350 nm) at which 5-HIAA is estimated. However, at the dose levels studied, carbonylmethyl-THBC does not interfere since it is extracted together with 5-HT before separation and estimation of 5-HIAA. Unlike 5-HT in acid solution, carbonylmethyl-THBC does not shift emission peak to 540 nm after excitation at 295 nm and thus does not interfere with the estimation of 5-HT.

Drugs studied were harmaline (Fluka AG), pargyline hydrochloride (Abbott Laboratories), imipramine hydrochloride and chlorimipramine hydrochloride (Ciba-Geigy), fluoxetine hydrochloride (Lilly Laboratories) and *d*-amphetamine sulfate (S.K. & F.).

Carbonylmethyl-THBC was one of a series of tetrahydro-β-carbolines synthesized by the reaction of 1,3-dicarbonyl compounds with L-tryptophan and tryptamine [23]. A suspension of tryptamine hydrochloride (Fluka AG, 0.392 g, 2 m-moles) in dimethylformamide (2 ml) was cooled in an ice bath and stirred magnetically. Triethylamine (0.278 ml, 2 m-moles) and then ethyl acetoacetate (0.253 ml, 2 m-moles) were added and stirring was continued at room temperature for 1 day. After the addition of ethyl acetate (20 ml) and aqueous sodium bicarbonate (1 M, 20 ml), the organic layer was separated, washed with bicarbonate solution and water, and dried over sodium sulfate. Evaporation yielded the intermediate enamine as a gum, which was directly acidolyzed with methanolic hydrogen chloride (1 M, 4 ml) at room temperature for 10 min. Evaporation to dryness, followed by treatment with ether, gave the product as a colorless solid which was recrystallized from butan-2-ol as needles m.p. 203–204.5° dec. (Found: C, 61.9; H, 6.5; Cl, 11.2; N, 9.1%. $C_{16}H_{21}ClN_2O_2$ requires C, 62.2; H, 6.9; Cl, 11.5; N, 9.1%). The results obtained by infra-red and p.m.r. spectroscopy support the assigned structure.

Carbonylmethyl-THBC increased the concentration of serotonin in mouse brain, without significantly altering the concentration of noradrenaline, dopamine, or the serotonin metabolite, 5-HIAA (Table 1). The increase in brain serotonin was probably not due to inhibition of MAO activity because carbonylmethyl-THBC is not a potent inhibitor of brain MAO activity *in vitro* or *ex vivo* when serotonin and tyramine are the substrates studied (Table 2). Moreover, MAO inhibition *in vivo* should result in a decrease in the brain concentration of 5-HIAA, as observed after harmaline (Table 1).

Carbonylmethyl-THBC was a weak inhibitor of the uptake of [^{3}H]5-HT and [^{3}H]NA into synaptosomes prepared from the mouse and rat brain (Table 3). Chlorimipramine and fluoxetine were greater than 10 times more potent than carbonylmethyl-THBC as inhibitors of [^{3}H]5-HT uptake.

At concentrations as low as 1 μm, carbonylmethyl-THBC increased the release of serotonin from synaptosomes prelabeled with [^{3}H]5-HT (Table 4). Although the basal release from mouse cerebral cortical synaptosomes was high, carbonylmethyl-THBC significantly increased both temperature dependent and K^+ evoked release of [^{3}H]5-HT. The effect of carbonylmethyl-THBC on the release of [^{3}H]5-HT was more obvious from synaptosomes prepared from the rat cerebral cortex. In this preparation, carbonylmethyl-THBC had a potency similar to that of amphetamine in releasing [^{3}H]5-HT.

Carbonylmethyl-THBC, although weaker, mimicked serotonin as an agonist on the isolated rat fundus preparation. The maximal response of the isolated tissue to both agonists was the same with the concentration of carbonylmethyl-THBC and serotonin required to produce 50 per cent of the maximal

Table 2. Effect of carbonylmethyl-THBC on MAO activity *

	Inhibition of MAO			
	In vitro K_i (μM)		*Ex vivo* IC_{50} (mg/kg, i.p.)	
Substrate	5-HT	Tyramine	5-HT	Tyramine
Carbonylmethyl-THBC	>50	>50	>100	>100
Harmaline	0.2	35	2	2
Pargyline	18	8.0	24	24

* MAO activity was determined in homogenates of whole mouse brain as detailed in Materials and Methods. For *ex vivo* studies, mice were killed 30 min after drug administration. The brain was removed, homogenized and MAO activity determined at substrate concentrations of 10^{-4} M.

Table 3. Effect of carbonylmethyl-THBC on synaptosomal uptake*

	Inhibition of uptake IC_{50} (μM)			
	Mouse		Rat	
	[^{3}H]5-HT	[^{3}H]NA	[^{3}H]5-HT	[^{3}H]NA
Carbonylmethyl-THBC	0.60	1.4	0.75	2.9
Imipramine	0.13	1.4	0.16	0.05
Chlorimipramine	0.01	1.3	0.02	0.05
Fluoxetine	0.06	5.5	0.07	1.2

*Uptake of [^{3}H]5-HT (5×10^{-8} M) and [^{3}H]NA (10^{-8} M) into synaptosomes prepared from rat cerebral cortex was determined as detailed in Materials and Methods.

contraction being 3 μM and 6 nM respectively. Methysergide (3 μM) completely blocked the response of both agonists.

If reproduced *in vivo*, the serotonin agonist activity of carbonylmethyl-THBC, together with the effects on serotonin release and uptake, would lead to a potentiation of central serotonergic activity. Such a potentiation could relate to the observed increase in brain serotonin concentration after carbonylmethyl-THBC if autoregulation of central serotonergic neurones occurs, as has been suggested to explain the accumulation of brain serotonin caused by drugs such as *d*-lysergic acid diethylamide [24–26]. Therefore, we plan to study the effect of carbonylmethyl-THBC *in vivo* on the neurophysiology of raphe neurones in the rat brain.

In summary, the effect of a novel tetrahydro-β-carboline, carbonylmethyl-THBC, on the neurochemistry of serotonin in the mouse brain has been studied. Carbonylmethyl-THBC increased brain serotonin levels but was not an inhibitor of monoamine oxidase activity. It increased serotonin release and weakly inhibited serotonin uptake into synaptosomes prepared from the rat or mouse cerebral cortex. Carbonylmethyl-THBC mimicked serotonin as an agonist on the isolated guinea pig ileum but was 500 times less potent.

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Table 4. Effect of carbonylmethyl-THBC on the release of serotonin*

	Release of serotonin (ratio [^{3}H]5-HT released:retained)	
	Mouse	Rat
Blank (0°)	0.26 ± 0.01†	0.09 ± 0.01†
Control (37°)	0.45 ± 0.01	0.24 ± 0.01
Carbonylmethyl-THBC (10^{-6} M)	0.72 ± 0.03†	0.51 ± 0.02†
(10^{-5} M)	0.78 ± 0.02†	0.64 ± 0.02†
Harmaline (10^{-6} M)	0.44 ± 0.02	0.26 ± 0.02
(10^{-5} M)	0.46 ± 0.02	0.28 ± 0.02
Amphetamine (10^{-6} M)	0.53 ± 0.01†	0.36 ± 0.01†
(10^{-5} M)	0.68 ± 0.03†	0.60 ± 0.02†
K^+ (60 mM)	1.05 ± 0.02†	0.70 ± 0.02†
K^+ (60 mM) + carbonylmethyl-THBC (10^{-5} M)	1.38 ± 0.04†	0.93 ± 0.02†
K^+ (60 nM) + harmaline (10^{-5} M)	1.27 ± 0.05†	0.73 ± 0.02†
K^+ (60 mM) + amphetamine (10^{-5} M)	1.37 ± 0.05†	1.06 ± 0.03†

*The release of serotonin was studied in synaptosomes prepared from mouse and rat cerebral cortex and prelabeled with [^{3}H]5-HT. Each value is the mean ± S.E.M. from at least six determinations.

† Significance of difference from mean control value (Student's *t*-test), $P < 0.001$.

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Degradation and re-synthesis of injected liver cadmium-thioneins in rat kidney

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Metallothionein has been considered to be a protective protein for the toxicity of heavy metals [1]. Contrary to the assumed biological role, Nordberg *et al.* [2], Cherian *et al.* [3], and Webb and Etienne [4] demonstrated the selective and more apparent toxicity of the injected metallothionein to renal tubular lining cells compared to cadmium ion. Recently we have compared the lesions induced by the injection of metallothioneins with differing Cd/Zn ratios and the time-dependent changes of the distribution patterns of cadmium, zinc, and copper among protein fractions in the kidneys after the injection [5]. From the results we suggested that the lesion induced by the injection of metallothionein is not due to metallothionein itself but due to the cadmium ion liberated from the degraded metallothionein in the kidneys.

The present study was intended to confirm that the injected metallothionein is degraded in the kidneys very shortly after the injection and that the liberated cadmium ion from the degraded protein induces the biosynthesis of metallothionein in the kidneys.

Cadmium-thionein-I and -II were prepared by replacing zinc in rat liver metallothionein with cadmium and separating on Sephadex G-75 and on DEAE Sephadex A-25 columns as reported already [5, 6]. The concentration of each cadmium-thionein solution was adjusted to 47.5 μg Cd/ml (Zn and Cu, less than 1 μg/ml; concentration of Tris–HCl buffer solution, 10–15 mM) by ultrafiltration on a Diaflo UM-10 membrane (Amicon). Each cadmium-thionein solution was injected intraperitoneally into 15 female rats of the Wistar strain (body wt, 135.7 ± 3.4 g, mean ± S.D.), respectively. The animals (5 rats/group) were killed 30 min, 1, and 3 days after the injection.

The metallothionein obtained from the kidneys of rats killed 30 min after the injection of cadmium-thionein-I or -II was separated into two forms on a DEAE Sephadex A-25

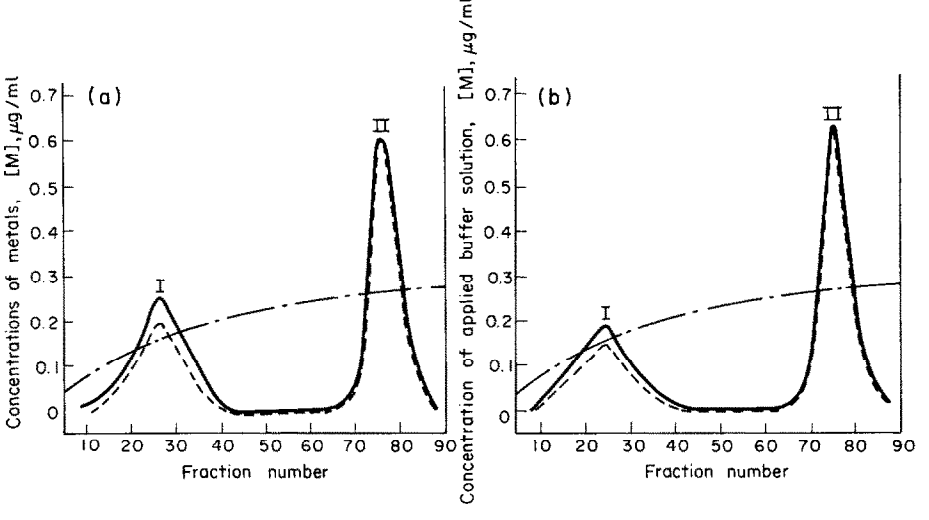

Fig. 1. DEAE Sephadex A-25 elution profiles of kidney metallothioneins obtained three days after the injection of cadmium-thionein-I (a) or -II (b). Five rats in each group were sacrificed 3 days after the injection of cadmium-thionein-I or -II. The kidneys in each group were combined, homogenized in three times the volume of 0.1 M Tris buffer solution (pH 7.4, 0.25 M glucose) using a Teflon homogenizer, and centrifuged at 105,000 *g* for 90 min at 2–4°. Each supernatant fraction was applied to a Sephadex G-75 column (5 × 80 cm) and eluted with 1 mM Tris buffer solution (pH 8.6). The metallothionein fraction was applied to a DEAE Sephadex A-25 column (1.5 × 28 cm) without concentration (100–120 ml). Two forms of metallothionein were eluted by a concentration gradient of Tris buffer solution (pH 8.6) between 1 mM (100 ml) and 300 mM (300 ml) after washing with 1 mM Tris buffer solution (pH 8.6) and collected (2.5 ml/tube). Metals were analyzed by a Hitachi 508 Atomic Absorption Spectrophotometer in each eluate. The curves are as follows: ——, Cd; — —, Zn; and — · —, applied buffer solution.