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## **THYMOL ESTER OF GAMMA-AMINO BUTYRIC ACID: SYNTHESIS AND ANTICONVULSANT ACTIVITY**

### **Abstract**

Thymol ester of gamma-aminobutyric acid (GABA) – 2-isopropyl-5-methylphenyl 4-aminobutyrate hydrochloride – was synthesized via Steglich esterification and characterized by <sup>1</sup>H NMR, IR and mass spectral studies. The anticonvulsant activity of obtained compound was estimated over a wide range of doses 5-80 mg/kg by determining the minimum effective doses of pentylenetetrazole inducing clonic-tonic convulsions and tonic extension. Present findings indicate that thymol ester of GABA is not a classical prodrug and possesses its own pharmacological activity. Prolonged antiseizure action of thymol derivative (20 mg/kg) was revealed at 24 hours after oral administration. Furthermore, orally co-administered gidazepam (1 mg/kg) and thymol ester of GABA (20 mg/kg) produce synergistic effect in seizures prevention.

**Key words:** thymol, gamma-aminobutyric acid, ester, anticonvulsant activity.

## **1. INTRODUCTION**

$\gamma$ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system and is widely used in the medicine and pharmacy. GABA neurotransmission alteration causes many neurological and psychiatric disorders such as epilepsy, cerebral ischemia etc. which require appropriate correction. GABA and other biologically active substances can be used as therapeutic agents. Currently there are many natural products of vegetable origin which affect the function of ionotropic GABA receptors; the main chemical classes of natural products presented in flavonoids, terpenoids, phenols, alcohols, and polyacetylene [1]. Thymol is a natural substance in the composition of the essential oil of thyme and a positive allosteric modulator of the GABA<sub>A</sub> receptors [2]. At higher concentrations, thymol has a direct action on GABA<sub>A</sub> receptors similar to that of the anesthetic propofol and other phenols [3]. Thymol induces a concentration-dependent increase in flunitrazepam binding in primary cultures of cortical neurons. The increase of GABA function and the blocking of voltage-gated sodium channels by thymol are resulted in sedative, anaesthetic and anti-epileptic pharmacological properties of this compound [4].

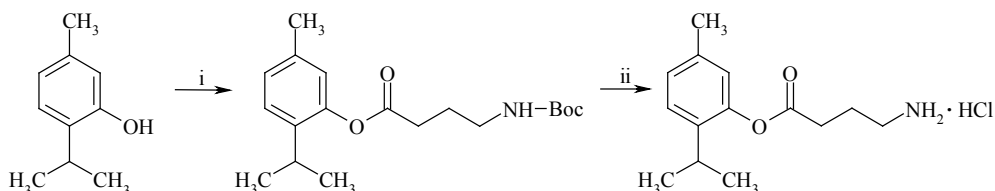
Thus, the aim of present work is synthesis of thymol-GABA ester as potential allosteric modulator GABA<sub>A</sub> receptor, as well as studying its anticonvulsant activity by using model experiments.

## **2. RESULTS AND DISCUSSION**

### *2.1. Synthesis*

Synthesis of thymol ester was carried out via Steglich esterification with N,N'-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as a catalyst

in dichloromethane (DCM). The condensation of 1.0 equivalent of thymol with 1.05 equivalent of Boc-protected GABA in the presence of DCC (1.1 equiv) and DMAP (catalytic amount) in dry DCM gave desired ester as yellow oily liquid (Scheme). The *tert*-butyloxycarbonyl (Boc) group was removed with 1 M HCl in glacial acetic acid according to the procedure [5].



**Scheme.** Synthesis of thymol-GABA ester. Reagents and conditions: (i) Boc-GABA, DMAP,  $\text{CH}_2\text{Cl}_2$ , rt, 10 min; DCC,  $0^\circ\text{C}$ , 30 min; rt, 10 h; (ii) HCl,  $\text{CH}_3\text{COOH}$ .

Synthesized thymol ester was fully characterized by  $^1\text{H}$  NMR, IR and FAB-mass spectrometry. The FAB-MS spectrum of obtained compound displays the protonated molecular ion peak  $[\text{M}+\text{H}]^+$  at  $m/z$  236. The IR spectrum of thymol ester exhibits absorption bands of N–H bond at  $3430\text{ cm}^{-1}$ , C=O ester group at  $1723\text{ cm}^{-1}$ , aromatic C–H at  $3330\text{ cm}^{-1}$  and alkyl C–H in the range  $2929\text{--}2851\text{ cm}^{-1}$ . The  $^1\text{H}$  NMR spectrum reveals one singlet at  $\delta$  2.21 ppm (3H), one doublet at  $\delta$  1.06 ppm ( $J = 6.77\text{ Hz}$ , 6H), and one multiplet at  $\delta$  2.79–2.86 ppm (1H) assigned to the protons of methyl and isopropyl groups. The aromatic ring protons appear as two doublets at  $\delta$  7.18 ppm ( $J = 7.03\text{ Hz}$ ) and 6.99 ppm ( $J = 7.28\text{ Hz}$ ) as well as one singlet at  $\delta$  6.80 ppm (1H each). The remaining signals of amino acid ester are observed at  $\delta$  1.74, 1.87–1.92, 2.29 ppm for protons of  $\gamma\text{-CH}_2$ ,  $\beta\text{-CH}_2$  and  $\alpha\text{-CH}_2$  groups, respectively (2H each).

## 2.2. Pharmacology

Chemical seizure models are widely used in the preclinical evaluation of drugs' anticonvulsant properties [6]. Bearing in mind the fact that thymol is a positive allosteric modulator of  $\text{GABA}_\text{A}$  receptors [2] the convulsive behavior of experimental animals was induced by convulsant pentylenetetrazole (PTZ) which is GABA receptor agonist. Thymol ester of gamma-aminobutyric acid was screened for its anticonvulsant potential through PTZ test that represents a valid model for human generalized myoclonic seizures [7].

Recently anticonvulsant and antiepileptogenic potential of thymol has been studied in different experimental models over a wide range of doses 5–100 mg/kg [8]. Based on these data, thymol ester in similar doses 5–80 mg/kg body weight was administered to mice orally in Tween 80/water emulsion, and Tween 80/water emulsion was used as a vehicle control. At the first stage of pharmacological researches dose-response relationship for thymol ester was established at 6 hours after a single oral administration (fig. 1). As seen, synthesized compound demonstrates dose dependent anticonvulsant action over a range of doses 5–20 mg/kg that is expressed as increasing of PTZ dose required for onset of clonic-tonic seizures (DCTC) and tonic extension (DTE) in experimental animals. Dose of 30 mg/kg caused anticonvulsant effect with DCTC and DTE values 203% and 205%, respectively, compared to vehicle treated control group. We should point out that 30 mg/kg is the ceiling dose since further increasing of ester dose up to 80 mg/kg body weight

has not affected the antiseizure potency in experimental animals: DCTC and DTE values remain constant (there is no statistically significant difference).

Taking into account these results, it has been proposed that thymol-GABA ester is not a classical prodrug and possesses its own pharmacological activity. In order to validate this assumption obtained ester at the dose of 20 mg/kg was examined at short time period – 1 h after oral administration that enables to prevent ester hydrolysis (table). At this time point DCTC and DTE values were found to be 263% and 256%, respectively, indicating 2.5 times protection against PTZ-induced seizures compared to control; therefore, our findings support the conclusion that thymol-GABA ester is active before it undergoes an enzymatic transformation *in vivo* to release parent terpene and amino acid.

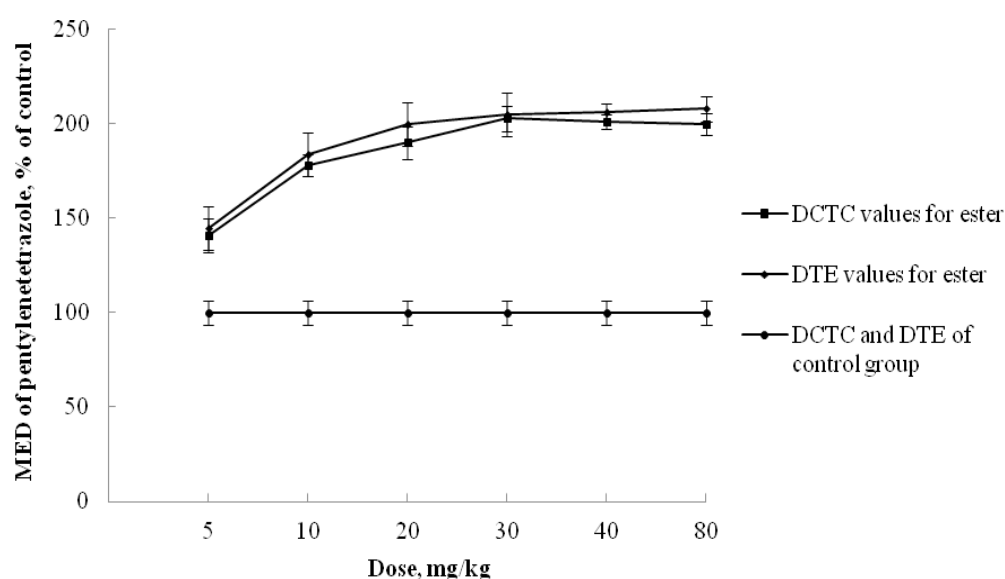


Fig. 1. Dose-response relationship of thymol-GABA ester at 6 h after oral administration. Values are given as mean  $\pm$  SEM for groups of five mice in each.

Considering the possible prolonged action of obtained ester, anticonvulsant evaluation was carried out at several additional time points – 3, 6 and 24 hours after single oral administration (20 mg/kg). This enables the pharmacokinetics of synthesized compound to be expressed as a function of time. According to our data thymol ester significantly and time dependently inhibited the onset of convulsions throughout the whole time period. As shown in Table ester treatment 24 h prior to PTZ intravenous administration produced antiseizure activity with DCTC and DTE values 181% and 183%, accordingly; thus, our experimental data provide strong evidence that synthesized compound possesses prolonged anticonvulsant action due to partial ester bond hydrolysis.

A large fraction of anticonvulsants is based on the attempt to boost inhibitory synaptic transmission in order to restore the balance between inhibition and excitation in epileptic tissue. Today, there are at least three different targets of anticonvulsant drugs at the synaptic level, all centered on the main inhibitory transmitter – GABA [9]. One of the most powerful anticonvulsants – benzodiazepines (BDZ), being GABA receptor

modulators, potentiate the effect of GABA by binding to the benzodiazepine site on GABA<sub>A</sub> receptor complex [10].

Table

Anticonvulsant action of thymol-GABA ester at dose of 20 mg/kg (time-response relationship)

MED of pentylenetetrazole, % of control					
Time, h	1	3	6	24	Control
DCTC	263±10.3	213±10.0	191±3.3	181±5.0	100±6.0
DTE	256±11.5	214±6.7	208±8.8	183±15.0	100±4.7

Values are given as mean ± SEM for groups of five mice in each.

Since the GABA<sub>A</sub> receptor is recognized as an important target for anticonvulsant drugs and due to both benzodiazepines and thymol are modulators of this receptor, in the present work we have also investigated co-administration effect of gidazepam (GDZ) and thymol-GABA ester. Gidazepam (1 mg/kg), thymol ester of GABA (20 mg/kg) and their mixture were administered orally at 3 h before PTZ intravenous injection.

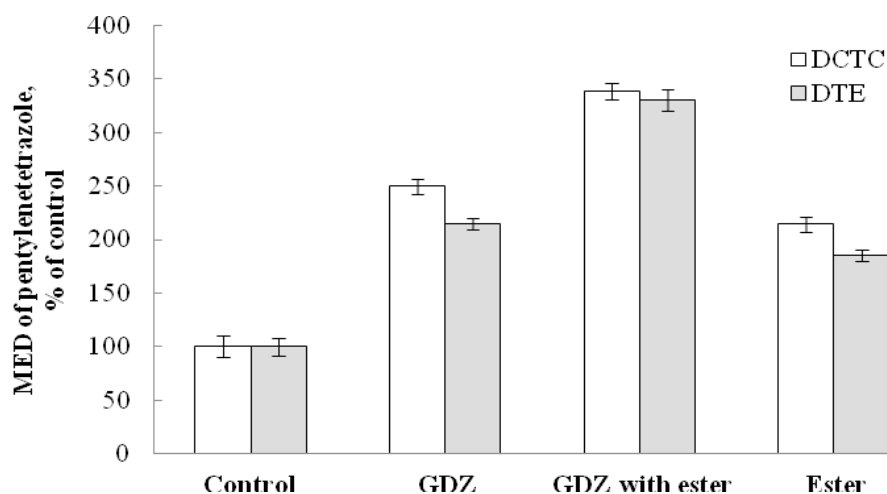


Fig. 2. Anticonvulsant activity of gidazepam (GDZ), thymol-GABA ester and their mixture. Values are given as mean ± SEM for groups of five mice in each.

As illustrated in fig. 2, both gidazepam and thymol ester of GABA reveals antiseizure effect in 3 h after oral administration with DCTC and DTE values: 250 and 215% for gidazepam; 214 and 185% for synthesized ester. Co-administration of GDZ and thymol-GABA ester was shown to increase anticonvulsant activity compared with each compound alone with DCTC and DTE values: 339 and 330%. These data demonstrate that orally co-administered gidazepam and thymol ester of GABA produce synergistic effect in seizures prevention suggesting that our compound as well as thymol is not

acting *via* the benzodiazepine site; these results are in agreement with those obtained by electrophysiological investigation [2].

### 3. EXPERIMENTAL SECTION

#### 3.1. Synthesis

Structure of obtained compound was established by  $^1\text{H}$  NMR spectroscopy on a Bruker AVANCE DRX 500 (500 MHz) using  $\text{DMSO-}d_6$  as a solvent and TMS as an internal standard. FAB mass spectrum was obtained on a VG 70-70EQ mass spectrometer equipped with Xe ion gun (8 kV); the sample was mixed with *m*-nitrobenzyl-alcohol matrix. IR spectrum was measured with a Perkin-Elmer FT-IR Spectrometer Frontier using KBr pellets. The purity and identity of the compound were monitored by TLC on Merck-made (TLC Silica gel 60 F<sub>254</sub>) plates.

#### *Synthesis of 2-isopropyl-5-methylphenyl 4-aminobutyrate hydrochloride*

To a stirred solution of thymol (0.5 g, 3.2 mmol) in  $\text{CH}_2\text{Cl}_2$  (20 mL) at the room temperature Boc-protected GABA (0.662 g, 3.26 mmol) and 4-dimethylaminopyridine (DMAP) (0.097 g, 0.794 mmol) were added. Reaction mass was cooled to  $0^\circ\text{C}$ , stirred for 10 min, and  $\text{N,N}'$ -dicyclohexylcarbodiimide (DCC) was added dropwise (0.727 g, 3.53 mmol). Stirring was continued for 30 min, then the flask was gradually warmed to the room temperature and the stirring continued for additional 10 h. Reaction completion was monitored by TLC. Reaction mixture was filtered, the filtrate was diluted to 100 mL and washed with 1 M aqueous HCl, 10% aqueous  $\text{NaHCO}_3$ , and water. Deprotection of the N-Boc group was carried out using  $\text{HCl}/\text{CH}_3\text{COOH}$  according to the procedure [5].

Yield: 80%. White solid. IR  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3430, 3330, 2929-2851, 1723, 1577, 1244.  $^1\text{H}$  NMR ( $\text{DMSO-}d_6$ , 500 MHz,  $\delta$ , ppm): 7.18 (d,  $J = 7.03$  Hz, 1H, Ar-H), 6.99 (d,  $J = 7.28$  Hz, 1H, Ar-H), 6.80 (s, 1H, Ar-H), 2.79–2.86 (m, 1H, CH), 2.29 (t, 2H,  $\alpha\text{-CH}_2$ ), 2.21 (s, 3H,  $\text{CH}_3$ ), 1.87–1.92 (m, 2H,  $\beta\text{-CH}_2$ ), 1.74 (t, 2H,  $\gamma\text{-CH}_2$ ), 1.06 (d,  $J = 6.77$  Hz, 6H,  $\text{CH}_3$ ). MS (FAB)  $m/z$ : 236 [ $M+\text{H}$ ] $^+$ .

#### 3.2. Pharmacology

Anticonvulsant activity of synthesized compound was studied using outbred male white mice (18-22 g) as experimental animals. They were kept under 12-hour light regime and in a standard animal facility with free access to water and food, in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Specific Purposes (Strasbourg, 1986) and the principles of the National Ukrainian Bioethics Congress (Kyiv, 2003). All the animals were purchased from Odessa National Medical University, Ukraine. The animal ethics committee of the Odessa National University (Ukraine) approved the study.

The anticonvulsant activity of tested compound was evaluated by pentylenetetrazole model (PTZ), which includes the determining of pentylenetetrazole minimum effective doses (MED) inducing clonic-tonic convulsions (CTC) and tonic extension (TE) in test animals upon intravenous infusion of 1% aqueous solution into a tail vein. Doses of pentylenetetrazole for inducing clonic-tonic convulsions (DCTC) and tonic extension (DTE) were calculated relative to control. The anticonvulsant effect of compounds was estimated at certain time points (1, 3, 6 and 24 hours) from the increase of pentylenetetrazole MED compared with a control group.

#### 4. Conclusion

Thymol ester of gamma-aminobutyric acid (GABA) – 2-isopropyl-5-methylphenyl 4-aminobutyrate hydrochloride – was synthesized in good yield, characterized by different spectral studies and its anticonvulsant potency was assessed against PTZ-induced seizures. Dose-response relationship was revealed for obtained compound over a range of doses 5-20 mg/kg. Synthesized thymol derivative orally administered at dose of 20 mg/kg exhibits time-dependent anticonvulsive protection; its activity is maintained for 24 hours that is an evidence of prolonged action. Moreover, co-administration of aforementioned ester and GABA receptor modulator – gidazepam leads to synergistic effect in seizures prevention.

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**ТИМОЛОВИЙ ЕСТЕР ГАММА-АМИНОМАСЛЯНОЇ КИСЛОТИ: СИНТЕЗ ТА ВИВЧЕННЯ ПРОТИСУДОМНОЇ АКТИВНОСТІ**

Синтезовано естер на основі тимолу та гамма-аміномасляної кислоти (ГАМК) – 2-ізопропіл-5-метилфеніл 4-амінобутират гідрохлорид; його будову підтверджено методами ІЧ-, <sup>1</sup>Н ЯМР спектроскопії та мас-спектрометрії. Протисудомна активність отриманої сполуки вивчена *in vivo* в інтервалі доз 5-80 мг/кг шляхом визначення мінімальних ефективних доз пентилентетразолу, що викликають клоніко-тонічні судоми та тонічну екстензію. Представлені дані свідчать, що синтезований тимоловий естер ГАМК не відноситься до класичних проліків та демонструє власну фармакологічну активність. Виявлено, що отримане похідне в дозі 20 мг/кг проявляє пролонговану протисудомну дію; при сумісному введенні з гідазепамом в дозі 1 мг/кг спостерігається ефект синергізму.

**Ключові слова:** тимол, гамма-аміномасляна кислота, естер, протисудомна активність.

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**ТИМОЛОВИЙ ЕФИР ГАММА-АМИНОМАСЛЯНОЇ КИСЛОТИ: СИНТЕЗ І ИЗУЧЕНИЕ ПРОТИВОСУДОРОЖНОЇ АКТИВНОСТІ**

Синтезовано складний ефір на основі тимолу та гамма-аміномасляної кислоти (ГАМК) – 2-ізопропіл-5-метилфеніл 4-амінобутират гідрохлорид; його будову підтверджено методами ІК-, <sup>1</sup>Н ЯМР спектроскопії та мас-спектрометрії. Противосудорожна активність отриманого сполука вивчена *in vivo* в інтервалі доз 5-80 мг/кг шляхом визначення мінімальних ефективних доз пентилентетразолу, що викликають клоніко-тонічні судороги та тонічну екстензію. Представлені дані свідчать, що синтезований тимоловий ефір ГАМК не є класичним пролекарством і має власну фармакологічну активність. Обнаружено, що отримане похідне в дозі 20 мг/кг проявляє пролонгированное противосудорожное действие; при совместном введении с гидазепамом в дозі 1 мг/кг спостерігається ефект синергізму.

**Ключевые слова:** тимол, гамма-аміномасляна кислота, складний ефір, противосудорожна активність.



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