Full Paper

Synthesis, Ultra-Short Acting Hypnotic Activity, and Metabolic Profile of Ethyl 8-Oxo-5,6,7,8-tetrahydro-thiazolo[3,2-*a*][1,3]diazepin-3-carboxylate (HIE-124)

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The synthesis and biological evaluation of ethyl 8-oxo-5,6,7,8-tetrahydro-thiazolo[3,2-*a*][1,3]diazepin-3-carboxylate (HIE-124, **4**), as a member of a new generation of ultra-short acting hypnotics is described. HIE-124 **4** exhibited potent *in-vivo* activity with a very rapid onset of action and a shorter duration of action with no acute tolerance or noticeable side effects than thiopental sodium. The rat *in-vivo* and *in-vitro* metabolic profile of **4** is also described. Urine was pooled from a number of animals and analyzed using electrospray liquid chromatography mass spectrometry (ESI LC-MS). HIE-124 **4** was incubated with rat-liver microsomal and rat-liver hepatocyte preparations then similarly analyzed. The only metabolic product of both *in-vitro* and *in-vivo* experiments is the carboxylic acid derivative **5**. HIE-124 **4** has the potential use not only as a pre-anesthetic medication and as anesthesia inducer but also has the potential to be used with thiopental sodium to maintain anesthesia for longer duration.

Keywords: Metabolic profile / Synthesis / Thiazolo[3,2-*a*][1,3]diazepine / Ultra-short acting hypnotic

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Introduction

Surgical procedures require the administration of several intravenous drugs to ensure hypnosis, analgesia, relaxation, and control of visceral reflex responses. The use of intravenous drugs adds flexibility and permits the administration of lower doses of inhalational anesthetic agents. General anesthesia most often is initiated by an injection of thiopental, an ultra-short acting barbiturate, to induce sleep prior to administration of the agents that are necessary for maintaining anesthesia during the surgical procedure. Ultra-short acting barbiturates and benzodiazepines have an important place in the practice of anesthesiology. Thiopental sodium remains the standard for comparison with new agents.

Single intravenous anesthetic dose of thiopental produces unconsciousness within 10 – 20 seconds. The depth of anesthesia may increase for up to 40 seconds then decreases progressively until consciousness returns in 20 – 30 minutes. However, recovery may require many hours if a large dose of thiopental sodium is administered [1]. Thiopental is metabolized slowly in the liver, which together with other factors, may influence the depth of anesthesia, time of recovery, and duration of action [2]. The concentration employed of thiopental should not exceed 2.5% in aqueous solution. When greater concentration is injected extravascularly, severe

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Abbreviations: ethyl 8-oxo-5,6,7,8-tetrahydro-thiazolo[3,2-*a*][1,3]diazepin-3-carboxylate (HIE-124); total ion current (TIC); rat liver microsomes (RLM's); glutathione (GSH); Troglitazone (TGZ); single ion monitoring (SIM)

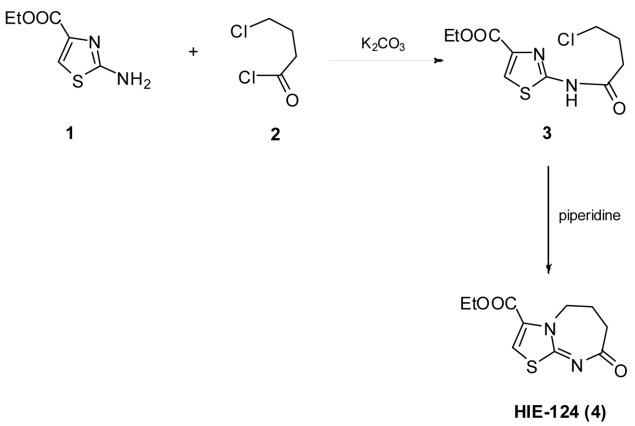
pain and tissue necrosis may occur. Intra-arterial injection of a concentrated solution of thiopental may result in damage to the arterial endothelium, followed by endarteritis, often with thrombosis exacerbated by subsequent arteriolar spasm. Vascular ischemia and even gangrene may result [3]. Thiopental produces a long lasting hangover [4]. Patients using a large initial dose of thiopental will awake despite plasma concentrations that normally would cause sleep. The nature of this acute tolerance is not known. For this reason, thiopental cannot be used to maintain surgical anesthesia, but only as an induction agent. Thiopental produces a dose-related depression of respiration that can be profound [5 – 7]. Larger doses of thiopental sodium cause more profound changes and respiration is maintained only by movements of the diaphragm.

Benzodiazepines were first introduced for the treatment of anxiety, then, later on, they have been used as sedatives, anticonvulsants, and muscle relaxants. Hypnosis and unconsciousness may be produced with large doses. Benzodiazepines are not analgesics, nor can they produce a state of surgical anesthesia when used alone. It is necessary to combine several drugs to achieve surgical levels of anesthesia with a balance of sedation, analgesia, amnesia, relaxation, and freedom from reflex stimulation. Benzodiazepines are useful as the sole agent for procedures that do not require analgesia, such as endoscopy, cardiodepression, cardiac catheterization and a spectrum of radiodiagnostic procedures. For induction of anesthesia, the benzodiazepines are given intravenously, to minimize the burning sensation and avoid venous thrombosis that might accompany the i.v. administration [8].

In the present investigation, we would like to report the ultra-short acting activity of ethyl 8-oxo-5,6,7,8-tetrahydro-thiazolo[3,2-*a*][1,3]diazepin-3-carboxylate (HIE-124, **4**), a member of a novel class which might overcomes many of the disadvantages and problems that are usually associated with the use of thiopental or benzodiazepines as intravenous anesthetic agents. HIE-124 **4** has been shown to not only induce anesthesia but also maintain the anesthetic state during the surgical procedure. HIE-124 **4** exhibited a very rapid onset of action and a shorter duration of action with no acute tolerance or noticeable side effects when compared with thiopental sodium. This new finding granted the issuance of a German patent [9].

Chemistry

The thiazolo[3,2-*a*][1,3]diazepine nucleus, can be obtained by the use of published methods [10]. Ethyl 8-oxo-5,6,7,8-tetrahydro-thiazolo[3,2*a*][1,3]-diazepin-3-carboxylate (HIE-124, **4**) was synthesized according to an inventive method (Scheme 1). Ethyl 2amino-thiazole-4-carboxylate **1**, was prepared using a reported procedure [11], and then alkylated with 4-chloro-butyryl chloride **2**, in presence of anhydrous potassium carbonate to afford ethyl 2-(4-chlorobutanamido)-thiazole-4-carboxylate **3**. Compound **3** was cyclized-using piperidine as a base to produce HIE-124 **4**, which was purified using silica gel column chromatography $CHCl_3$/hexane (80 : 20 *v*/*v*). Structure elucidation of compounds **3** and **4** was obtained based on analysis of the ^{1}H-, ^{13}C-, DEPT-, COSY-, HMBC-, and HMQC-NMR spectra, in addition to mass spectrometry and elemental analyses for each compound.



Scheme 1. Synthesis route of presented compound HIE-124 (**4**).

Results and discussion

Intravenous administration of ethyl 8-oxo-5,6,7,8-tetrahydro-thiazolo[3,2-*a*][1,3]diazepin-3-carboxylate (HIE-124, **4**), at dose levels of 50 and 100 mg/kg, induced hypnosis

Table 1. Hypnotic effect of ethyl 8-oxo-5,6,7,8-tetrahydro-thiazolo[3,2-*a*][1,3]diazepin-3-carboxylate (HIE-124, **4**), 50 and 100 mg/kg IV, and its effect on the thiopental sodium, 50 mg/kg IV, – induced hypnosis in mice.

Treatment	Dose i.v. [mg/kg]	Sleeping time [min]
Thiopental Sodium	50	4.90 ± 1.13
HIE-124 (**4**)	50	0.75 ± 0.01*
HIE-124 (**4**)	100	1.48 ± 0.21*
HIE-124 (**4**) + Thiopental	50 + 50	19.90 ± 5.21*
HIE-124 (**4**) + Thiopental	100 + 50	25.70 ± 5.11*

Data are expressed as means ± S.E., n = 10.

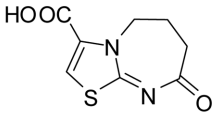
* Significantly different as compared to thiopental, using student's t-test, $P < 0.05$.

Table 2. Hypnotic effect of ethyl 8-oxo-5,6,7,8-tetrahydro-thiazolo[3,2-*a*][1,3]diazepin-3-carboxylate (HIE-124, **4**),100 and 200 mg/kg IP and its effect on the thiopental sodium, 65 mg/kg IP, induced hypnosis in mice.

Treatment	Dose i.p. [mg/kg]	Sleeping time [min]
Thiopental Sodium	65	5.2 ± 1.40
HIE-124 (**4**)	100	2.4 ± 0.83*
HIE-124 (**4**)	200	9.1 ± 1.23*
HIE-124 (**4**) + Thiopental	100 + 65	49.2 ± 3.71*
HIE-124 (**4**) + Thiopental	200 + 65	155.7 ± 16.35*

Data are expressed as means ± S.E., n = 10.

* Significantly different as compared to thiopental, using student's t-test, $P < 0.05$.



5

Figure 1. Structure of the major metabolite **5**.

in all mice within few seconds (Table 1). Tachypnea was observed in three mice out of ten receiving 100 mg/kg; also dyspnea was observed in one mouse out of ten mice receiving the same dose (100 mg/kg). Both doses of HIE-124 **4** potently potentate the hypnotic effect produced by 50 mg/kg i.v. thiopental sodium (Table 1). I.p. administration of HIE-124 **4** at dose levels of 100 and 200 mg/kg, induced hypnosis in all mice with an onset of action in less than one minute (Table 2). Tachypnea was observed in two mice out of ten receiving 100 mg/kg, and ataxia and tachypnea were also observed in three mice out of ten mice receiving 200 mg/kg. Both doses of the compound, 100 and 200 mg/kg, potently potentated the hypnotic effect produced by 65 mg/kg i.p. thiopental sodium [12], (Table 2).

Acute toxicity test was performed according to the method described by Litchfield and Wilcoxon [13]. The calculated LD_{50} HIE-124 **4** was found to be 681.4 mg/kg with 95% confidence limits of 482.8 – 876.4. An acute tolerance test was conducted following the i.p. administration of 200 mg/kg of HIE-124 **4**, three times a day for three consecutive days. There was no significant difference among the sleeping times after the first and the last administration of the compound. The sleeping time after the first administration of **4** was 10.3 ± 1.4 min., while after the ninth administration, the sleeping time was 8.7 ± 2.1 minutes.

Initial ESI LC-MS analysis of HIE-124 **4** (Fig. 1) dissolved in 60 : 40 $CH_3CN : H_2O$ showed the compound eluting at ~17 minutes with a protonated molecular ion peak $[M + 1]^+$ at m/z 241 in the positive ion mode (Fig. 2). Gradient elution was tailored such that HIE-124 **4** elutes at a relatively late analysis time to allow for a time window sufficient for the elution of other metabolites, which are expected to be polar, and, hence, elute at an earlier time. Screening for potential metabolites was performed by searching for ions with molecular weights corresponding to metabolites generated through one of the most common phase-I and phase-II metabolic transformations.

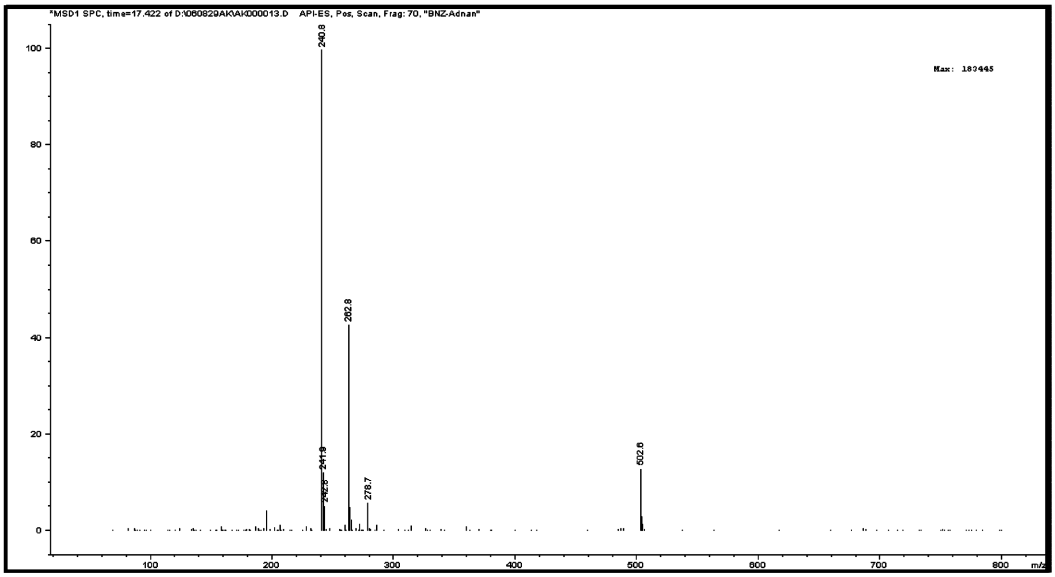


Figure 2. ESI mass spectrum of HIE-124 (**4**) dissolved in 60 : 40 $CH_3CN : H_2O$.

Table 3. Summary of RLM experiments conducted.

Incubation type	Microsomes	HIE-124 (4)	NADPH	$MgCl_2$	Buffer
Test	X	X	X	X	X
Control 1	X	X	0	X	X
Control 2	0	X	X	X	X

X = addition; 0 = no addition.

Table 4. RLMs incubation volumes.

Incubation type	Microsomes	HIE-124 (4)	NADPH	$MgCl_2$	Buffer
Test	80 µL	1 µL	100 µL	100 µL	719 µL
Control 1	80 µL	1 µL	–	100 µL	819 µL
Control 2	–	1 µL	100 µL	100 µL	799 µL

For HIE-124 **4**, these transformations were expected to be ester hydrolysis, aromatic hydroxylation, and glucuronic acid conjugation. The primary strategy for metabolite identification was thorough analysis of total ion current (TIC) chromatograms obtained from various LC-MS analyses. Blank TIC and UV chromatograms were subtracted from those containing compound **4** in either a urine sample or an *in-vitro* incubation.

Screening of TIC chromatograms of pooled mice-urine samples showed a prominent peak eluting at ~11 minutes, with a protonated molecular ion peak at m/z 213, with the absence of the peak at m/z 241 corresponding to the protonated **4** (Fig. 2). The peak was proposed to be the de-ethylated metabolite **5** $[M - 28]^+$, resulting from hydrolysis of the ethyl ester of **4**. It is a well documented route of biotransformation [14–17]. Careful screening of the TIC chromatogram showed the absence of any other peak corresponding to one of the expected metabolites resulting from either phase-I reactions (*e.g.* m/z 229 resulting from hydroxylation) or phase -I reactions (*e.g.* m/z 389 resulting from glucuronidation).

In-vitro drug metabolism studies are widely regarded as reliable and accurate models for the prediction of drug metabolism in a variety of mammalian species. Among the different techniques to study *in-vitro* metabolism are hepatic microsomal models and hepatocyte preparations [18, 19]. This study attempted to utilize such techniques to gain more information about the metabolic profile of HIE-124 **4**. Incubation of compound **4** with rat liver microsomes (RLM's) (Tables 3 and 4) was straightforward and results were consistent with those obtained from *in-vivo* experiments. The various incubation conditions were designed to obtain more details about the possible enzymatic processes involved in the *in-vitro* metabolism of **4**. Incubations with NADPH alone with no cells were performed to determine the presence of non-enzymatic hydrolysis of the ester. Incubation with cells but no NADPH was designed to determine involvement of non-CYP450 isoforms in the metabolism of **4** since CYP450 isoforms need NADPH for the catalysis. Incubation with glutathione (GSH) (Table 5) was designed to determine the presence of GSH adducts, frequently formed with carboxylic acids [20–21]. Troglitazone (TGZ), known to form GSH adducts [22], was used as a positive control.

LC-MS analysis of the *in-vitro* studies gave similar and consistent results to the *in-vivo* experiments. In RLM studies, the only metabolite detected for HIE-124 **4** was the de-ethylated product **5** $[M - 28]^+$ at m/z 213 (Fig. 3). The chromatogram showed no evidence of the parent compound after the conclusion of the incubation time (Fig. 4). No

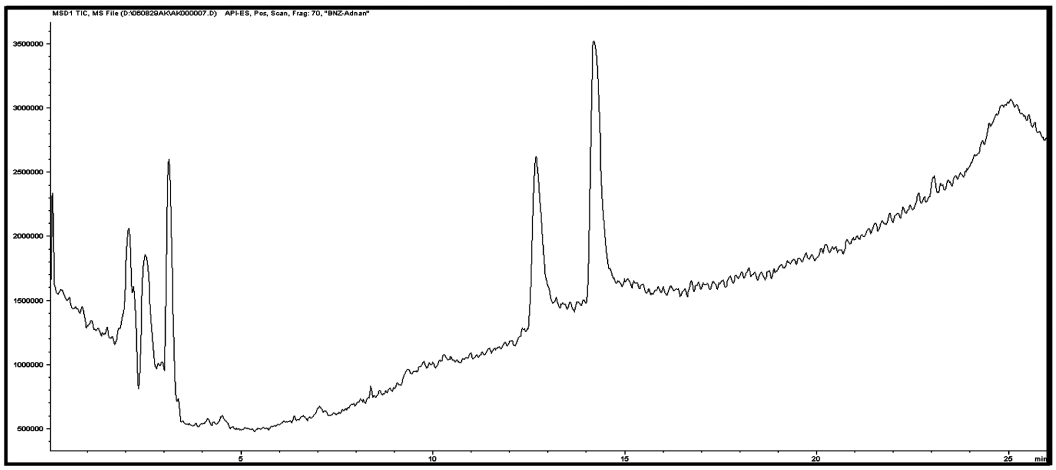


Figure 3. TIC chromatogram of blank hepatocyte incubation.

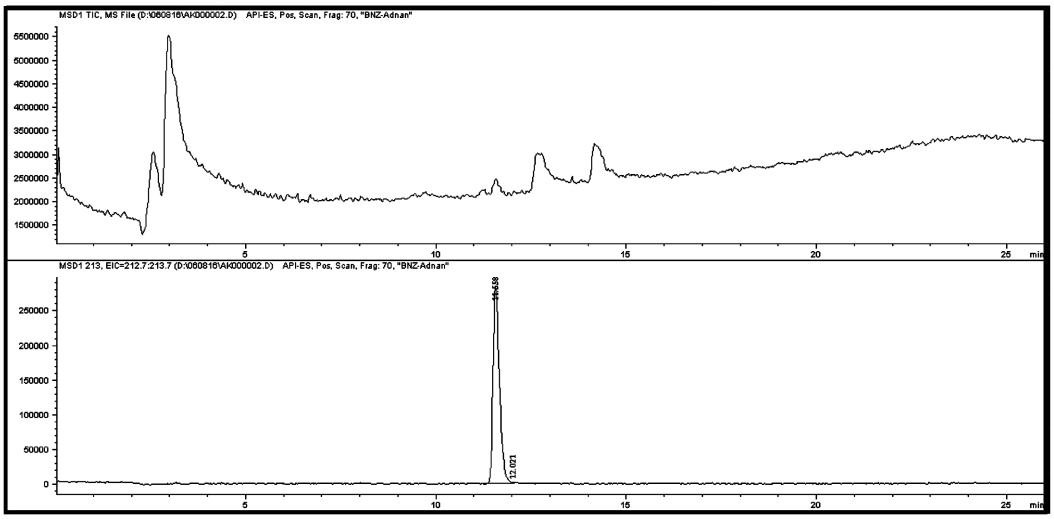


Figure 4. TIC and SIM (m/z 213) chromatograms of 30 min RLM incubation with HIE-124 (**4**).

Table 5. GSH incubations.

Incubation type	Microsomes	HIE-124 (4) or TGZ	NADPH	$MgCl_2$	GSH	Buffer
Test	107 μL	1 μL	100 μL	100 μL	100 μL	592 μL
Control 1	107 μL	1 μL	–	100 μL	–	792 μL
Control 2	107 μL	1 μL	–	100 μL	100 μL	692 μL
Control 3	107 μL	1 μL	100 μL	100 μL	–	692 μL
Control 4	107 μL	–	100 μL	100 μL	–	693 μL

other drug-related peaks were detected in the run compared to a blank TIC chromatogram. Compound **5** was also the only metabolic product observed for **4** after hepatocyte incubations (Fig. 5). The compound appears to be the product of enzymatic hydrolysis by the action of liver esterases rather than a simple chemical hydrolysis as confirmed by the absence of any significant amounts of the hydrolyzed product, as in the case of RLM and hepatocyte studies. Furthermore, the involvement of esterases in the hydrolysis is supported in part by the presence of the hydrolyzed product in RLM incubations, which does not contain NADPH, the necessary co-factor for cytochrome P450-mediated metabolism but not for esterase-mediated hydrolysis. The presence of carboxyesterase activity in freshly thawed hepatocytes was confirmed by the carboxyfluorescein diacetate assay, frequently used to easily determine cell-derived enzymatic esterfication [23 – 25]. HIE-124 **4** appeared to undergo rapid hydrolysis to the corresponding carboxylic acid derivative, with undetected levels of the parent compound remaining in aliquots untreated with CH_3CN taken at zero minute hepatocyte incubation time. RLM incubations of **4** in the presence of GSH did not result in the formation of GSH adducts based on positive ion ESI LC-MS results.

Conclusion

The biological evaluation of HIE-124 **4** revealed that the compound is a very short acting hypnotic. The onset of action of this compound is very short compared to thiopental sodium. In all animals used, the onset of action was less than 60 seconds. The duration of action when given i.v. or i.p. is significantly shorter than that of thiopental sodium. In addition, the investigated compound did not show any sign of acute tolerance reported with the second (maintenance) dose of thiopental sodium. Therefore, compound HIE-124 **4** has a potential use not only as a pre-anesthetic medication and induction of anesthesia but also has the potential to be used with thiopental sodium to maintain anesthesia for longer duration than using thiopental sodium alone. Compound **4** appears to undergo enzymatic hydrolysis by the action of liver esterases. Further metabolic studies are needed to

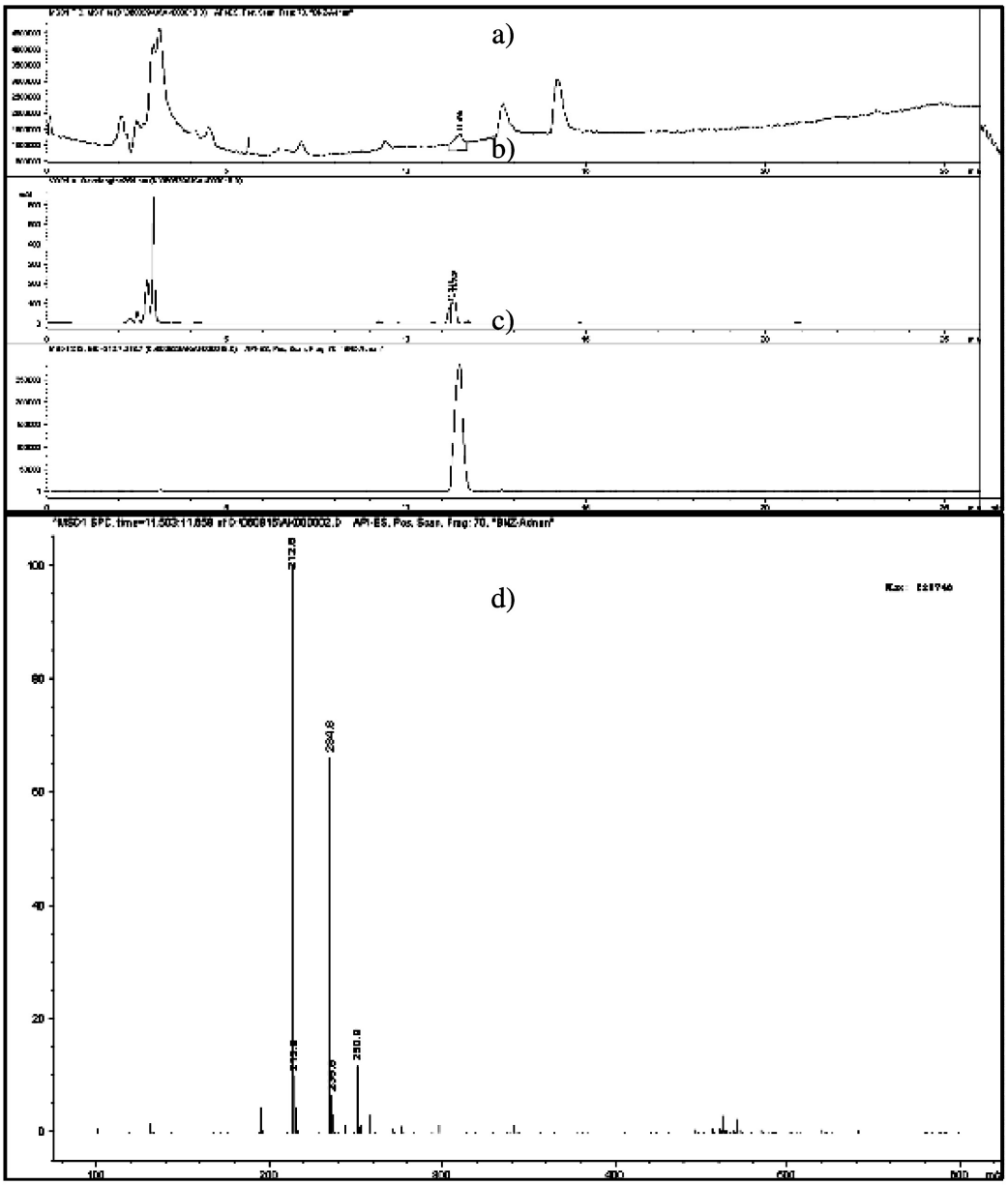


Figure 5. Chromatograms of hepatocyte incubation with HIE-124 (**4**) after 120 minutes.
a) TIC; b) UV; c) SIM (m/z 213); d) ESI mass spectrum of **5**.

characterize the metabolism of this compound from other mammalian systems.

The authors have declared no conflict of interest.

Experimental

Melting points (°C) were determined on Mettler FP80 melting point apparatus (Mettler-Toledo, Greifensee, Switzerland) and are uncorrected. Microanalyses were performed on a Perkin Elmer 240 elemental analyzer (Perkin Elmer, Beaconsfield, UK) at the Institut of Pharmacy, Friedrich-Schiller-University Jena, Germany. All of the new compounds were analyzed for C, H, and N and agreed with the proposed structures within ± 0.4% of the theoretical values. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL 500 MHz FT spectrometer (Varian Inc., Palo Alto, CA, USA); chemical shifts are expressed in δ ppm with reference to TMS. Mass spectral (MS) data were obtained on a Shimadzu GC/MS QP 5000 apparatus (Shimadzu, Tokyo, Japan). Thin layer chromatography was performed on precoated (0.25 mm) silica gel GF_{254} plates (E. Merck, Darmstadt, Germany), compounds were detected with 254 nm UV lamp. Silica gel (60 – 230 mesh) was employed for routine column chromatography separations.

Ultra-short acting hypnotic evaluation experiments were performed at Pharmacology Department, College of Pharmacy, Mansoura University, Egypt. Analytical chromatography separation and analytes' mass determination was carried out on an Agilent 1100 MSD® (Agilent, Palo Alto, CA, USA) system consisting of an Agilent 1100 series HPLC system equipped with an ultraviolet detector and interfaced via electrospray ionization (ESI) to an Agilent 1100 single quadrupole mass spectrometer. The chromatographic separation was carried out on a Hypersil C18 250 × 3.0 ID, 5 μm particle size column, eluting with a mobile phase consisting of A: 1% formic acid and B: acetonitrile (CH_3CN) in a linear gradient program running at a constant flow rate of 0.5 mL/min. The program was started at 5% (CH_3CN) which was linearly increased to 60% over 20 minutes, kept at 20% for 2 minutes, then returned to 5% B in one minute and kept at the composition for the final 3 minutes. UV absorbance was monitored at 254 nm. MS experiments were carried out in the positive mode, using nitrogen as nebulizing gas at 300. Total ion current (TIC) as well as single ion monitoring (SIM) experiments were conducted to screen for the possible metabolites. TIC experiments were conducted in the range from 100 – 600 amu, and SIM experiments were set to monitor for a number of ions including m/z 241 $[M + H]^+$, m/z 213 $[M - 28]^+$, m/z 229 $[M + 16]^+$, m/z 389 $[M + 176]^{+\bullet}$ CH_3CN and formic acid were all HPLC grade. NADPH, $MgCl_2$ were obtained from Sigma, Inc. (U.K). All buffer solutions were prepared in-house.

Chemistry

Ethyl 2-(4-chlorobutanamido)-thiazole-4-carboxylate ***3***

A mixture of ethyl 2-aminothiazole-4-carboxylate (**1**, 7.0 g, 0.04 mol) and 4-chloro-butyryl chloride (**2**, 11.3 g, 9.0 mL, 0.08 mol) in toluene (100 mL) was heated under reflux for 4 h. The toluene was then evaporated under reduced pressure. Then, the residue was quenched with water, stirred, and filtered. The solid obtained was washed, dried, and recrystallized from water to give the required product **3** (10.2 g, 92%): m.p. 171 – 172°C; ^{1}H-NMR ($CDCl_3$) d 1.27 (t, 3H, *J* = 7.0 Hz, C*H*$_3$CH$_2$), 2.02 – 2.08 (m, 2H, *J* = 6.5, 7.3 Hz, ClCH$_2$C*H*$_2$CH$_2$N), 2.59 (t, 2H, *J* = 7.3 Hz, Cl(CH$_2$)$_2$C*H*$_2$N), 3.66 (t, 2H, *J* = 6.5 Hz, ClC*H*$_2$(CH$_2$)$_2$N), 4.27 (q, 2H, *J* = 7.0 Hz, CH$_3$C*H*$_2$), 7.96 (s, 1H, thiazoleH), 12.5 (brs, 1H, NH). ^{13}C-NMR δ 14.1, 27.7, 32.4, 44.9, 60.8, 122.7, 141.3, 158.3, 161.3, 171.3; MS *m/e* 276.0 (85). Anal. ($C_{10}H_{13}ClN_2O_3S$) C, H, N.

Ethyl 8-oxo-5,6,7,8-tetrahydro-thiazolo[3,2-a] [1,3]diazepin-3-carboxylate ***4***

A mixture of ethyl 2-(4-chlorobutanamido)-thiazole-4-carboxylate (**3**, 1.0 g, 0.004 mol) and piperidine (0.7 g, 0.8 mL, 0.008 mol) in toluene (50 mL) was heated under reflux for 3 h. The reaction mixture was cooled, poured into water, and stirred. Toluene was separated, dried, and evaporated to give a crude product, which was purified by repeated silica gel column chromatography eluting with $CHCl_3$/hexane (80 : 20 *v/v*) to give **4** (0.7 g, 80%): m.p. 210–212°C; ^{1}H-NMR ($CDCl_3$) δ 1.22 (t, 3H, *J* = 7.1 Hz, C*H*$_3$CH$_2$), 2.08-2.14 (m, 2H, *J* = 8.0, 4.5 Hz, COCH$_2$C*H*$_2$CH$_2$N), 2.54 (t, 2H, *J* = 8.0 Hz, COCH$_2$CH$_2$C*H*$_2$N), 4.07 (t, 2H, *J* = 4.5 Hz, COC*H*$_2$CH$_2$CH$_2$N), 4.19 – 4.23 (q, 2H, *J* = 7.1 Hz, CH$_3$C*H*$_2$), 7.7 (s, 1H, thiazoleH). ^{13}C-NMR δ 14.6, 18.3, 31.9, 48.3, 61.4, 122.7, 142.1, 157.7, 161.7, 174.3; MS *m/e* 240.0 (65) Anal. ($C_{10}H_{12}N_2O_3S$) C, H, N.

Ultra-short acting hypnotic evaluation

Mice of both sexes (22 – 28 g) were used to conduct the ultra-short acting hypnotic evaluation. Fifty and 100 mg/kg of compound HIE-124 **4** were injected intravenously into two groups of mice, respectively; each group consists of ten mice. The sleeping time was recorded for each mouse and compared with control ultra-short acting hypnotic agent. The effect of the test compound, administered intravenously, on thiopental sodium (Intraval sod. May & Baker LTD, England) -induced sleeping time was also evaluated. Three groups of mice each consists of ten mice were used. The first group (control), injected with the solvent"15% cremophore EL", the second group, treated with 50 mg/kg, and the third group, treated with 100 mg/kg of the test compound intravenously. All mice in the three groups were then injected with thiopental sodium, 50 mg/kg intravenously. The time elapsed between the loss and the recovery of righting reflex"sleeping time" was recorded for each animal and compared with control group (Table 1).

An amount of 100 and 200 mg/kg of compound HIE-124 **4** was injected intraperitoneally into two groups of mice, respectively. Each group consists of ten mice. The sleeping time were recorded for each mouse and compared with control ultra short-acting hypnotic agent. The effect of the test compound, administered intraperitoneally, on thiopental sodium-induced sleeping time was also evaluated. Three groups of mice, each consisting of ten mice were used. The first group (control), injected with the solvent"15% cremophore EL", the second group, treated with 100 mg/kg, and the third group, treated with 200 mg/kg compound of formula (**I**) intraperitoneally. All mice in the three groups were then injected with thiopental sodium; 65 mg/kg intraperitoneally [12]. The sleeping time was recorded for each animal and compared with control group (Table 2).

Acute toxicity test

Five groups of mice, each consisting of ten animals were used to conduct the acute toxicity test and to calculate the LD_{50}. Compound HIE-124 **4** dissolved in 15% cremophore EL was given intraperitoneally in doses of 100, 200, 400, 800, and 1600 mg/kg, respectively. The final volume of injection in all groups did not exceed 0.2 mL/mouse. Twenty-four hours later, the% mortality in each group was recorded and the LD_{50} was calculated using the method described by Litchfield and Wilcoxon [13].

Acute tolerance test

Ten mice were used to conduct the acute tolerance experiment. Each mouse received 200 mg/kg of compound HIE-124 **4** dissolved in 15% cremophore intraperitoneally, three times per day for three consecutive days. After each of the nine administrations, the sleeping time induced by the test compound was recorded for each mouse.

In-vivo metabolic studies

Male mice (n = 4, average ~25 g) were housed in special purpose metabolism cages obtained from Nalgene1. Animals will be acclimated in the cages for 48 hours prior to the start of the study in a 12 hour light/dark cycle (7:00 – 19:00 o'clock) and the animals were allowed free access to standard animal feed and water *ad-libitum*. HIE-124 **4** was formulated into a 20% ethanol solution. Each animal received a 30 mg/kg dose of the solution at 24, 72, and 96 hours. Urine draining into the special urine compartments fitted to the metabolism cages were collected

prior to drug dosing as blank control reference and at 24, 48, 72, 96, and 120 hours following drug dosing. Urine samples were pooled and stored at 4°C until analysis. Urine samples (20 mL) were filtered over 0.45 μm filters and passed through C18 SPE cartridges for purification. The cartridges were conditioned with methanol and water prior to loading of the urine sample. The samples were eluted with 2 mL methanol and the solvent was evaporated under nitrogen, and the residue was reconstituted in 300 μL of 60 : 40 CH_3CN : H_2O mixture and transferred to HPLC vials for analysis.

Rat liver microsomes (RLM's)

Male Sprague-Dawley rat (350 g) was obtained from the animal facility at Strathclyde University. RLM's were prepared by differential centrifugation [26]. The animal was incapacitated by cervical dislocation and, within no more than ten minutes, an incision was made in the peritoneal cavity, the liver excised and cut into small pieces, and suspended in ice-cold KCl/sucrose buffer. The liver pieces were then washed once with fresh KCl/sucrose buffer and homogenized in a 25 g liver/100 mL buffer ration using a standard homogenizer. Liver homogenate was transferred to centrifuge tubes and subjected to differential centrifugation in a Beckman Coulter® L8-55 M ultracentrifuge (Beckman, CA, USA) to obtain RLM's. Centrifugation was performed first at 10 800 rpm (~10 432 rcf) for 25 minutes and then discarding the supernatant. The resulting S9 fraction was resuspended in KCl/sucrose buffer (containing 0.04 M KH_2PO_4/NaH_2PO_4, 0.25 M sucrose, 0.15 M KCl, pH 7.4) and centrifuged again at 33 000 rpm (~97 400 rcf) for 65 minutes. The supernatant was discarded and pellets rinsed with KCl/sucrose buffer, pooled together and manually stirred to produce a homogenous microsomal suspension. Suspension was stored at −70°C for no longer than two weeks until use. Protein content of RLM's was determined according to the method of Lowry *et al.* [27]. Microsomal incubations were carried out according to the summary in Table 3. Aliquots of 1 mL each were used for the various types of experiments. Amounts added in each container are given in Table 4. The various components of the incubation reaction were added following a specific order. To the appropriate container was added an appropriate volume of ice-cold phosphate buffer (0.08 M KH_2PO_4/NaH_2PO_4, pH 7.4), an appropriate volume of an ice-cold freshly prepared $MgCl_2$ solution, 1 μL of a 100 M stock solution of HIE-124 **4** and an appropriate volume of the RLM suspension. Test tubes containing the incubation mixtures were pre-incubated at a temperature of 37°C in the water bath for 5 minutes. An appropriate volume of NADPH was then added after the incubation mixtures have reached 37°C. The incubation was allowed to run for 30 minutes, and the reaction was terminated by the addition of 2 mL of ice-cold CH_3CN. The mixture was centrifuged for ten minutes at 14 000 rpm and the supernatant transferred to a fresh container and subjected to evaporation under a stream of nitrogen. The residue was reconstituted in 300 μL of 60 : 40 CH_3CN : H_2O and transferred to HPLC vials for analysis.

GSH experiments

Glutathione (GSH) incubations were carried out in a way similar to the RLM incubations described above (see section 5.5.1). Protocol and amounts are shown in Table 5.

Rat liver hepatocyte preparation

Rat liver hepatocytes were freshly prepared following previously published and established rat-liver perfusion procedures [28–30]. Hepatocyte viability was checked prior to the start of the incubation and again at the conclusion. Viability of >75% was accepted to proceed with the incubation. Hepatocyte incubation experiments were carried out in a rotary evaporation apparatus fitted with a custom-made four-way adaptor to allow for the fitting of four 100 mL round bottom flasks. Each of the four flasks contained 12 mL of cell suspension. The first two flasks were used as controls, one containing HIE-124 **4** and the other none. The latter two flasks contained two different concentrations of HIE-124 **4** (50 μM and 100 μM, respectively). A 5% : 95% gaseous mixture of CO_2 and O_2 was continuously bubbled through the four flasks. The incubations were carried out at 37°C for 2 hours. An amount of 1 mL aliquots (at 0, 15, 30, 60, 90, and 120 minutes) was taken from each flask and mixed with two volumes of CH_3CN and were immediately flash-frozen in liquid nitrogen and stored at −70°C until analysis. To test esterase activity, two 1 mL aliquots (drawn at 0 minutes) were immediately flash frozen with no addition of CH_3CN and stored at −70°C until analysis. Samples were thawed and three volumes of CH_3CN were added to each thawed sample and vortex-mixed for 2 minutes, then centrifuged at 14 000 rpm. The supernatants were transferred to fresh containers and evaporated under a stream of nitrogen. The remaining residues were reconstituted in 300 μL of 60 : 40 CH_3CN : H_2O and transferred to HPLC vials for analysis.

Carboxyesterase activity assay

Frozen hepatocyte suspension (1 mL) was thawed and centrifuged at 14 000 rpm for 5 minutes to obtain a clear cell suspension. The suspension (990 μL) was incubated with 5(6)-carboxyfluorescein diacetate (CFDA, 1 μL of a 2.5 mM solution in DMSO) in a glass cuvette and fluorometric readings were taken every 30 seconds for 15 minutes at 479 and 580 nm excitation and emission wavelengths, respectively.

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