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Full Paper

Design and Synthesis of 1,2,4-Triazolo[3,2-*b*]-1,3,5-thiadiazine Derivatives as a Novel Template for Analgesic/Anti-Inflammatory Activity

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Previously, we demonstrated that certain heterocyclic compounds derived from 3-substituted-1,2,4-triazole-5-thiones had promising analgesic/anti-inflammatory activities together with low ulcerogenic properties. Therefore, we sought to design and synthesize new derivatives of triazol-5-thiones-fused heterocycles. In the present study, a series of novel *bis*-Mannich bases, namely 2,6-disubstituted-6,7-dihydro-5*H*-1,2,4-triazolo[3,2-*b*]-1,3,5-thiadiazines (**1a–d**, **2a–c**, and **3a–d**), were synthesized and characterized to assess their possible anti-inflammatory/analgesic properties. Additionally, their ability to induce gastric toxicity was also evaluated. Several of the condensed compounds produced a degree of analgesic activity comparable to reference drugs in both the hot plate and tail-flick tests. A strong anti-inflammatory effect was observed for the derivatives carrying a benzyl group at the second position (**2a–c**). The majority of the prepared compounds caused comparatively less gastrointestinal (GI) side effects than the reference drugs naproxen and indomethacin did. These results showed that 1,2,4-triazolo[3,2-*b*]-1,3,5-thiadiazine derivatives might afford a safer alternative to currently available analgesic/anti-inflammatory agents for the treatment and management of inflammatory disease and pain.

Keywords: 1,2,4-Triazolo[3,2-*b*]-1,3,5-thiadiazine / Analgesic activity / Anti-inflammatory activity / NSAIDs / Ulcerogenic risk

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Introduction

Inflammation is the response of the body to hazardous stimuli. The symptoms, warmth, pain, redness, and swelling, are caused by vascular and cellular changes. Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed categories of drugs worldwide for the treatment of pain and inflammation [1]. However, all NSAID treatment carries some risk of gastrointestinal (GI) toxicity, ranging in severity from mild dyspepsia to GI hemorrhage and perforation, and these side effects are mainly observed in cases of chronic NSAID usage [2]. Indeed, the most severe complications may lead to hospitalization, surgery, or death. Several strategies exist to reduce the incidence and frequency of NSAID-related GI complications [1], including the co-prescription of a histamine-2 receptor antagonist (H_2RA), proton pump inhibitor (PPI), or misoprostol. Alternatively, the prescription of a cyclooxygenase-2 (COX-2) preferential or specific

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NSAID rather than a conventional NSAID is also used [3]. Therefore, development of more effective NSAIDs endowed with an improved safety profile is still an attractive research area in medicinal chemistry. Recent studies have highlighted the importance of the synthesis and characterization of various 1,2,4-triazoles and their fused heterocyclic derivatives. Over the past 20 years, our interest has focused on the synthesis of novel heterocyclic systems with analgesic/anti-inflammatory activity. In this regard, the chemistry and the synthesis of mercapto-1,2,4-triazoles and several of their condensed derivatives, for example, thiazolo[3,2-*b*]-1,2,4-triazole (**I–IV**) [4–8], 1,2,4-triazolo[3,2-*b*]-1,3-thiazine (**V**) [9], 1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazine (**VI**) [10, 11], and triazolothiadiazole (**VII**) [12] (Scheme 1) have attracted our primary focus. Our research on the condensed 1,2,4-triazole compounds has yielded a significantly large number of compounds (**I–VII**) endowed with compelling analgesic/anti-inflammatory activity, as well as reduced gastrointestinal toxicity compared to classical NSAIDs.

A survey of the existing literature revealed that a *bis*-Mannich reaction of 5-mercapto-3-aryl-1,2,4-triazoles with primary amines presented a synthesis method for the 1,2,4-triazolo[3,2-*b*]-1,3,5-thiadiazine ring system. However, limited work has been previously performed regarding primary amines, and no attention has been paid to the analgesic/anti-inflammatory effects of these derivatives up to the present day [13–15]. Therefore, we aimed to synthesize novel *bis*-Mannich derivatives of 1,2,4-triazole-5-thiones, namely 2,6-disubstituted-6,7-dihydro-5*H*-1,2,4-triazolo[3,2-*b*]-1,3,5-thiadiazines (**1a–d**, **2a–c**, **3a–d**), and to investigate their possible analgesic/anti-inflammatory properties.

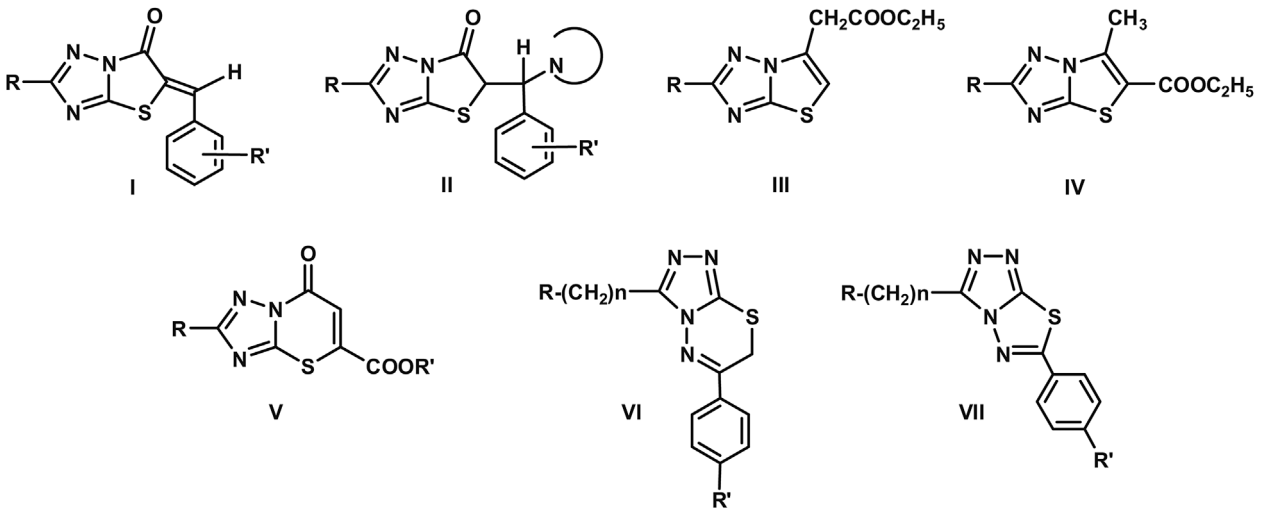
Results and discussion

Chemistry

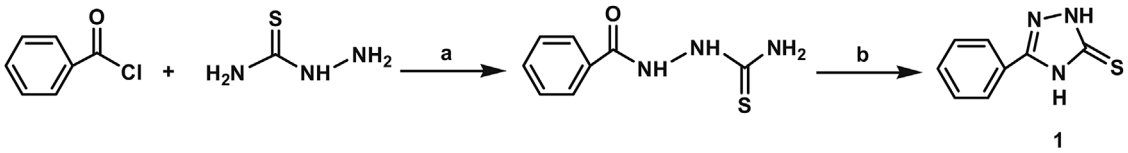
The synthesis of the starting compounds (3-aryl-/3-arylalkyl-1,2,4-triazole-5-thiones) was accomplished via various methods as illustrated in Schemes 2 and 3. The 3-phenyl substituted derivative (**1**) of the series was prepared by the reaction of benzoyl chloride with the thiosemicarbazide, while the 3-arylalkyl-1,2,4-triazole-5-thiones (**2** and **3**) were obtained via dicyclohexylcarbodiimide (DCC)-promoted amide formation reaction, starting from a carboxylic acid to the method reported earlier [4, 5, 7].

The compounds were then subjected to a Mannich reaction by treatment with formaldehyde and various primary amines, such as butyl- (**a**), benzyl- (**b**), 2-phenethyl- (**c**), and (−)-1-phenethylamine (**d**), in ethanolic medium. It was found that the reaction proceeds via cyclization, consuming 2 mol of formaldehyde and resulting in mild to good yields of the target 1,2,4-triazolo[3,2-*b*]-1,3,5-thiadiazines (Scheme 4).

The cyclized compounds **1a–d**, **2a–c**, and **3a–d** were characterized using IR, NMR, and mass spectral analysis. IR spectra of compounds showed no NH and C=S signals, providing evidence for the formation of a cyclic ring. In addition, ^{1}H-NMR confirmed the structures of the triazolo-[3,2-*b*]-1,3,5-thiadiazine derivatives **1a–3d** (except **1d** and **3d**) from the appearance of two singlet signals at about δ 4.56–4.90 and 5.05–5.22, which were attributed to the two methylene groups SCH_2N and NCH_2N, respectively, and the disappearance of the exchangeable protons at δ 12.97–13.86 ppm. Methylene protons of the thiadiazine ring in compounds **1d** and **3d** showed a prominent AB system with protons Ha and Hb, which were seen as separate doublets at δ 4.58, 4.85, 5.09, and 5.34 (J_{AB}: 12.5 Hz) with one hydrogen



Scheme 1. Chemical structures of selected heterocycles derived from 1,2,4-triazole previously reported as anti-inflammatory/analgesic agents.



Scheme 2. Synthesis of the 3-phenyl-1,2,4-triazol-5-thione. Reagents and conditions: (a) *i*-PrOH; (b) NaOH followed by acidification with concentrated HCl.

integration. In order to explain the interaction of protons with each other, advanced NMR techniques were performed for these two compounds. Examination of the HSQC (heteronuclear single-quantum correlation) NMR spectrum of compound **1d** showed that the peaks seen at δ 4.58, 4.85, and 5.09, 5.34 belonged to C-5 (S<u>C</u>H_2N) and C-7 (N<u>C</u>H_2N) carbons, respectively. It was assumed that because of the ring current effect of the phenyl ring, one of each pair of methylene protons (axial or equatorial) resonated at a much lower field (about 0.26 ppm). In the NOESY spectra, a NOE effect appeared between methyl and both deshielded methylene protons. According to the Dreiding model, methyl group is only oriented closer to the equatorial proton in *S* configuration of the stereogenic center. Thus, it has been suggested that the reaction proceeded while retaining this configuration, and that the reaction product had the same relative configuration as (−)-1-phenethylamine.

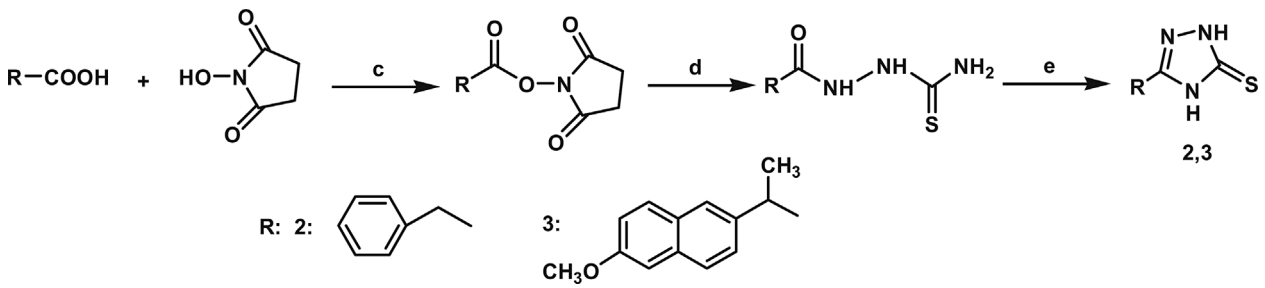
In the ESI-MS spectra, quasimolecular ion [M^{+}·+H] or [M^{+}·+Na] peaks, which appeared at different intensities, confirmed the molecular weights of the examined compounds. Although it is not possible to draw a common fragmentation pattern concerning all compounds, some generalizations could be made, and a representative fragmentation pattern, that of compound **2b**, is shown below (Scheme 5). The fragments resulting from the loss of the substituent at the second position, together with the attached carbon and additional sulfur atom from the molecular ion were observed for all compounds. Major fragmentation peaks (*m/z*: 57 for butyl, *m/z*: 91 for benzyl; *m/z*: 105 for 1-/2-phenethyl) formed the C–N bond cleavage at the sixth position of the condensed ring, and were seen at different intensities. Further spectroscopic details for these compounds are presented in the methods.

Pharmacology

Both the starting compounds **1–3** and their corresponding condensed derivatives (**1a–d**, **2a–c**, **3a–d**) were tested for anti-inflammatory and analgesic activity, as well as for ulcerogenic risk and toxicity upon acute administration. The analgesic activity of the compounds was evaluated by using both hot plate [16] and tail-flick tests [17]. In order to screen the anti-inflammatory profile, the carrageenan-induced hind-paw edema model in mice was used [18]. The gastric safety on acute administration was conducted via microscopic examination of the stomach. The compounds were applied at a dose of 30 mg/kg orally in the form of a suspension (0.5% carboxy methylcellulose as vehicle). Two nonselective COX inhibitors, naproxen (30 mg/kg), and indomethacin (10 mg/kg), and an opioid analgesic oxycodone (30 mg/kg), which acts via a different mechanism than the COX inhibitors, were utilized as reference drugs during pharmacological evaluation. Since indomethacin causes mortality at high doses, only 10 mg/kg was administered instead of 30 mg/kg.

Analgesic activity of the compounds: Hot plate test

In this test, the latency of paw-licking or a jump response was calculated for each animal as an index of nociception. All synthesized derivatives, except compounds **1–3**, significantly prolonged the latency of thermal-induced algesic responses compared to vehicle control. The highest analgesic activity



Scheme 3. Synthesis of 3-arylalkyl-1,2,4-triazole-5-thiones **2**, **3**. Reagents and conditions: (c) DCC/THF 0°C; (d) Thiosemicarbazide/THF; (e) NaOH followed by acidification with concentrated HCl.

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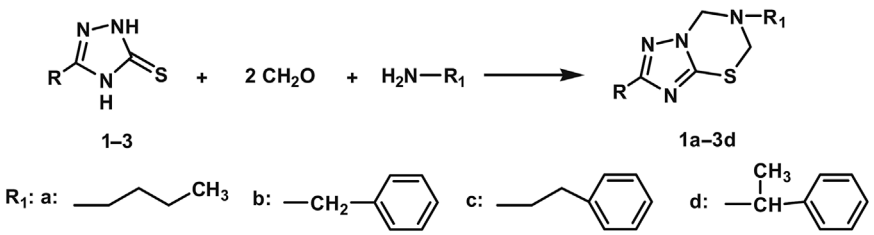


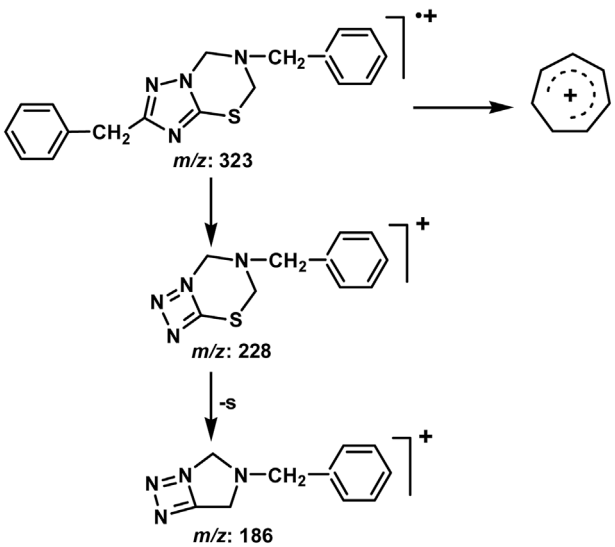
Scheme 4. Synthesis of 2,6-disubstituted-4,5,6,7-tetrahydro-1,2,4-triazolo[3,2-*b*]-1,3,5-thiadiazines (**1a–3d**).

was observed for derivatives **1a** and **3a**, containing a butyl group on the thiadiazine moiety. However, the introduction of a benzyl or phenethyl group at the sixth position of the ring decreased the activity (**1a** compared to **1b–d**; **3a** compared to **3b–d**) (Fig. 1).

Compounds **1a** and **3a,** compared to naproxen, showed the highest analgesic activity. Although **1d** did not reach the levels of **1a** and **3a**, it also had marked analgesic activity (Fig. 1).

Analgesic activity of the compounds: Tail-flick test

The thermal nociceptive threshold was assessed with a tail-flick test by measuring the latency to mouse tail withdrawal from the heat source. All compounds, except **1**, **2**, **3**, **2c**, and **3c**, produced some increased latency of the tail-flick response compared to control. Compounds **3a** and **3b** had the highest analgesic activity among these, with values of 28 and 31%, respectively. Compounds **1a**, **1b**, **2a**, and **3d** also showed moderate analgesic activity compared to naproxen (Fig. 2).



Scheme 5. A represent fragmentation pattern for 2,6-disubstituted-4,5,6,7-tetrahydro-1,2,4-triazolo[3,2-*b*]-1,3,5-thiadiazines.

On the basis of the results of the analgesic activity experiments, all compounds (except the starting compounds **1–3**) showed some degree of analgesic activity in both hot plate and tail-flick tests (Figs. 1 and 2). Although the results of the tail-flick and hot plate tests showed similar analgesic activity profiles, none of the test compounds reached the analgesic activities of the nonsteroidal analgesic naproxen and narcotic analgesic oxycodone. Among the compounds, the compounds **1a**, **3a**, and **3d** showed the most compelling analgesic activity in both tests.

Anti-inflammatory activity

The anti-inflammatory activity of the synthesized derivatives was assessed from their ability to inhibit hind paw edema induced by carrageenan, as evaluated by measuring paw thickness [18]. All compounds reduced carrageenan-induced hind-paw edema to different degrees than the vehicle control did. Compounds **1d**, **2a–c**, and **3d** showed the highest anti-inflammatory activity statistically, ranging from 48 to 58%, while naproxen and indomethacin showed 67 and 37% activity, respectively. When compared with naproxen, compounds **1a** and **3b** showed moderate anti-inflammatory activity (Fig. 3).

The highest anti-inflammatory activity was observed for derivatives **2a–c**, which had a benzyl at the second position of the ring. Compounds **2a** and **2b** were the most effective, displaying 58% inhibition value. Furthermore, **1d** and **3d**, with 1-phenethyl at the sixth position of the ring, had similar anti-inflammatory activity to naproxen.

When comparing the results of the analgesic and anti-inflammatory tests, a similar pattern of activity was observed regarding compounds **1**, **3**, **1a–c**, and **3a–d**. However, anti-inflammatory activity was more noticeable for compounds **2**, **1d**, and **2a–c** than analgesic activity.

Some preliminary conclusions could be drawn from these obtained results. The condensation of 1,2,4-triazole-5-thione (starting compounds **1–3**) with a thiadiazine ring increased both analgesic and anti-inflammatory activities. It seemed that the nature of the substituent at both the second and sixth positions affected the activity profile of the synthesized compounds. A significant difference in analgesic activity was observed depending on the substituent at the sixth position of triazolothiadiazine ring. In general, the compounds bearing a butyl **(a)** group at the sixth position showed higher analgesic activity than the others did. On the other hand, it

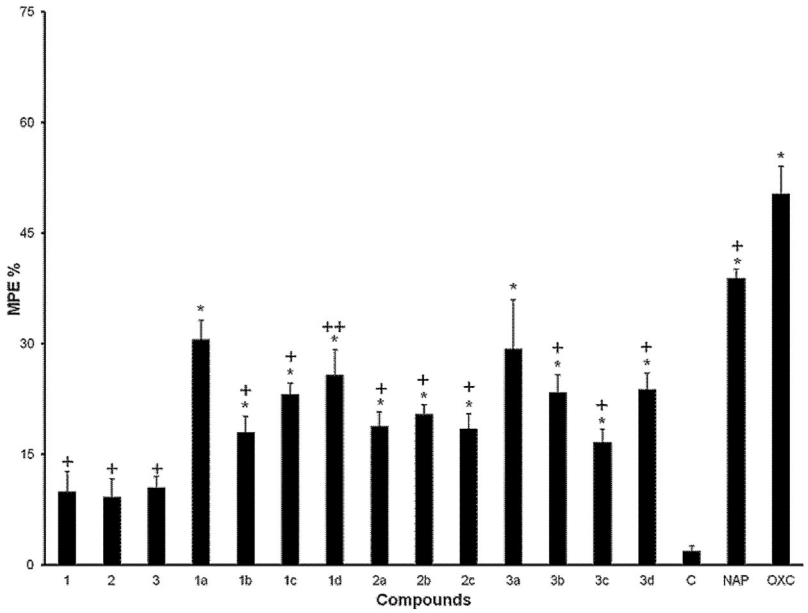


Figure 1. Analgesic activity results of the hot plate experiment for synthesized compounds, naproxen (NAP), and oxycodone (OXC) at 30 mg/kg doses. $^{*}p < 0.01$ versus vehicle control (C) group; $^{+}p < 0.01$ versus naproxen; $^{++}p < 0.05$ versus naproxen; data expressed as mean ± SEM, one-way ANOVA followed by *post-hoc* Dunnett's test, $n = 6$ per group.

was found that 2-benzyl analogs (**2a–c**) possessed enhanced anti-inflammatory properties compared to their corresponding 2-phenyl analogs (**1a–c**) and 2-[1-(naphthyl)ethyl] derivatives (**3a–c**).

Ulcerogenic liability

Nonsteroidal anti-inflammatory drugs exhibit significant ulcerogenic risk, which can be demonstrated in animal models. We therefore screened all synthesized compounds for adverse ulcerogenic effects. Naproxen (30 mg/kg) and indomethacin (10 mg/kg) were used as positive control, since they have been known to cause bleeding at higher doses. The stomachs of mice were examined for lesions in the gastric mucosa under a dissecting microscope. The quantification of gastric mucosal lesions was scored according to their numbers and size on a scale from 0 to 7 points, as detailed in the

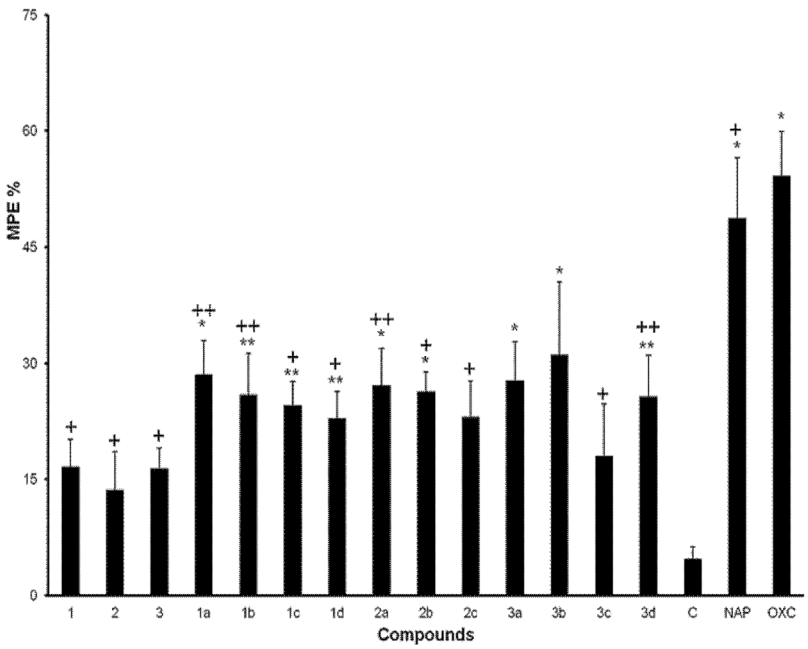


Figure 2. Analgesic activity results of the tail-flick experiment for the synthesized compounds, naproxen (NAP), and oxycodone (OXC) at 30 mg/kg dose. $^{*}p < 0.01$ versus vehicle control (C) group; $^{**}p < 0.05$ versus vehicle control; $^{+}p < 0.01$ versus naproxen; $^{++}p < 0.05$ versus naproxen; data expressed as mean ± SEM, one-way ANOVA followed by *post-hoc* Dunnett's test, $n = 6$ per group.

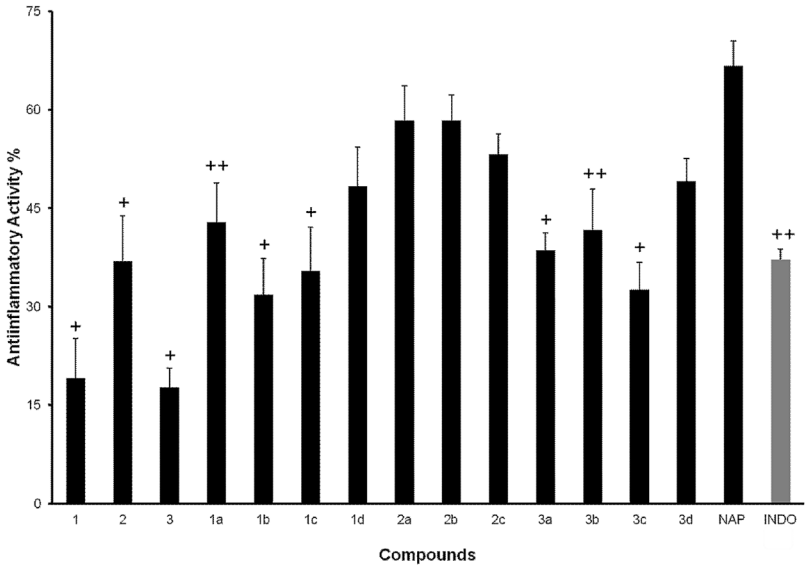


Figure 3. Anti-inflammatory activity results of the carrageenan induced hind paw edema experiment for the compounds (30 mg/kg), naproxen (NAP, 30 mg/kg), and indomethacin (INDO, 10 mg/kg, gray bar) $^{+}p < 0.01$ versus naproxen; $^{++}p < 0.05$ versus naproxen; data expressed as mean ± SEM, one-way ANOVA followed by *post-hoc* Dunnett's test, $n = 6$ per group.

methods section. In contrast to the high gastric ulcer incidence in naproxen and indomethacin, ulceration risk was significantly lower in our compounds except compound **3d**. In particular, compounds **1**, **3**, **1c**, **1d**, **2a**, **3a**, and **3b** provided superior GI tolerability statistically compared to naproxen. Although not statistically significant, the ulcerogenic scores of compounds **2**, **1a**, **1b**, **2b**, **2c**, and **3c** were also smaller than that of naproxen (Fig. 4).

Among the fused compounds, compounds **1a**, **1d**, **2a–c**, and **3b** showed marked anti-inflammatory and analgesic activity as well as lower ulcerogenic risk. Despite the fact that compound **3d** showed noticeable anti-inflammatory and analgesic activities, it caused a significant ulcerogenic effect on par with naproxen (Fig. 4).

Acute toxicity

In the acute toxicity study, mortality was not observed at 30 mg/kg doses. During the 14-day observation period, animals showed no weight loss, behavioral alteration, or signs of toxicity.

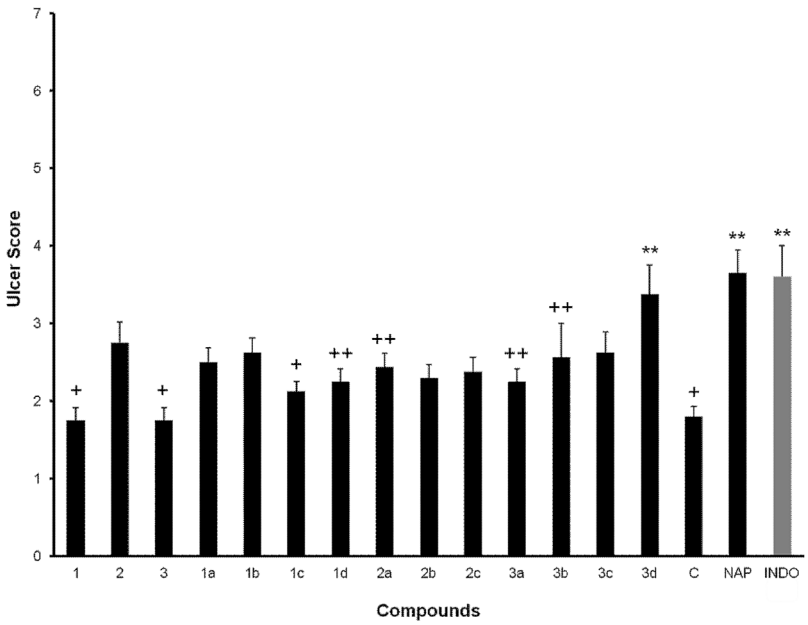


Figure 4. Ulcerogenic effect of synthesized compounds, naproxen (NAP, 30 mg/kg) and indomethacin (INDO, 10 mg/kg, gray bar). $^{**}p < 0.05$ versus vehicle control (C); $^{+}p < 0.01$ versus naproxen; $^{++}p < 0.05$ versus naproxen; data expressed as mean ± SEM, Kruskal–Wallis test followed by a *post-hoc* Dunn's test, $n = 6$ per group.

Conclusions

In the present study, 2,6-disubstituted-6,7-dihydro-5*H*-1,2,4-triazolo[3,2-*b*]-1,3,5-thiadiazine derivatives were prepared for the purpose of developing compounds with not only improved activity but also reduced gastric side effects. The compounds were synthesized via double Mannich reaction in one-pot synthesis. All synthesized compounds were screened for their analgesic/anti-inflammatory activity and ulcerogenic risk in animal models. The results were conclusive in showing that there is a noticeable increase in both analgesic and anti-inflammatory activity when mercaptotriazoles were condensed with a thiadiazine ring. Although our compounds showed less analgesic activity than we expected, they had marked anti-inflammatory activity with lower ulcerogenic risks compared to reference drugs. The derivatives carrying a benzyl group at the second position (**2a–c**) showed the most remarkable anti-inflammatory activities with reduced gastric side effects compared to reference drugs, while possessing moderate analgesic activity.

Experimental

Chemistry

Melting points (mp) were measured with a Thomas Hoover capillary melting point apparatus (Philadelphia, PA, USA) and are uncorrected. The $[\alpha]_D^{20}$ values were determined and calculated using a Rudolph Autopol IV Automatic Polarimeter (Flanders, USA). Infrared (IR) spectra were recorded on a PerkinElmer 1720X FT-IR spectrometer (Beaconsfield, UK) by using potassium bromide pellets, with the results expressed in wave number (cm^{-1}). ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-NMR spectra were taken on a Varian Mercury 400, 400 MHz High Performance Digital FT-NMR instrument (Palo Alto, CA, USA) in $CDCl_3$ using TMS as internal standard. All chemical shifts were recorded as δ (ppm) values. Splitting patterns are as follows: s, singlet; d, doublet; t, triplet; m, multiplet. The purity of the compounds was checked using silica gel-coated aluminum sheets (Merck, 1.005554, silica gel $HF_{254-361}$, type 60, 0.25 mm) via thin layer chromatography. The elementary analysis of new compounds was performed with a Leco CHNS 932 analyzer at Ankara University, Faculty of Pharmacy Central II Laboratory. Elementary analysis for C, H, N, and S were within $\pm$0.4% of theoretical values. All chemicals were from Aldrich Chemical Co. (Steinheim, Germany).

The InChI codes of the investigated compounds together with some biological activity data are provided as Supporting Information.

*Synthesis of 3-phenyl-1,2,4-triazole-5-thiones (**1**)*
Compound **1** was synthesized according to the previously reported procedure [4].

*Synthesis of 3-arylalkyl-1,2,4-triazole-5-thiones (**2**, **3**)*
Compounds **2** and **3** were prepared according to previously reported methods [5, 7].

*Synthesis of 2,6-disubstituted-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine derivatives (**1a–3d**)*
A mixture of 5 mmol 3-substituted-1,2,4-triazole-5-thione, 5 mmol appropriate primary amine (benzyl amine, buthyl amine, 2-phenylethylamine, (−)-1-phenylethylamine), and 1.2 mL formaldehyde was stirred in 10 mL ethanol for 5 h at room temperature. The mixture was poured into ice water and allowed to stand overnight. The precipitate was filtered. For the synthesis of compound **2c**, as well as the other compounds, 10 mmol triethylamine was added. The mixture was poured into acetic acid 5% and allowed to stand overnight. The precipitate was filtered. However, compound **2d** could not be obtained in this condition.

*6-Butyl-2-phenyl-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**1a**)*
Yield 84%, mp 80–81°C. ^{1}H NMR ($CDCl_3$): δ 0.94 (3H; t; -CH_2-CH_3), 1.38 (2H; m; -CH_2-CH_3), 1.57 (2H; m; -CH_2-CH_2-CH_2-), 2.81 (2H; t; -N-CH_2-CH_2-), 4.73 (2H; s; -S-CH_2), 5.17 (2H; s; -N-CH_2), 7.27–8.07 (5H; m; ArH). ^{13}C-NMR ($CDCl_3$): δ 14.07 (-CH_2-CH_3), 20.30 (-CH_2-CH_3), 29.62 (-CH_2-CH_2-CH_2-), 50.25 (-N-CH_2-CH_2-), 55.78 (*C*-5), 68.09 (*C*-7), 126.43 (Ph *C*-2, *C*-6), 128.79 (Ph *C*-4), 129.53 (Ph *C*-3, *C*-5), 130.79 (Ph *C*-1), 147.89 (*C*-3a), 160.70 (*C*-2). IR ν_{max} cm^{-1}: 3062 (C-H aromatic), 2956 (C-H aliphatic), 1608 (C=N), 1439 (C=C), 1130 (C-N), 633 (C-S). ESI-MS *m/z* (%): 297 (M^++Na, 30.77%), 275 (M^++H, 28.99%), 212, 190, 184, 170, 152 (100%), 108, 57. Anal. calcd. for $C_{14}H_{18}N_4S$: C, 61.28; H, 6.61; N, 20.42; S, 11.69. Found: C, 61.22; H, 6.72; N, 20.16; S, 11.57.

*6-Benzyl-2-phenyl-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**1b**)*
Yield 81%, mp 132–134°C. ^{1}H NMR ($CDCl_3$): δ 3.99 (2H; s; -N-CH_2-Ar), 4.66 (2H; s; -S-CH_2), 5.22 (2H; s; -N-CH_2), 7.26–8.09 (10H; m; ArH). ^{13}C-NMR ($CDCl_3$): δ 56.25 (-N-CH_2-Ph), 59.78 (*C*-5), 67.09 (*C*-7), 120.79 (Ph *C*-4), 127.43 (Ph *C*-2, *C*-6, *C*-4′), 128.95 (Ph *C*-2′, *C*-3′, *C*-5′, *C*-6′), 129.53 (Ph *C*-3, *C*-5), 130.79 (Ph *C*-1), 135.6 (Ph *C*-1′), 151.89 (*C*-3a), 160.70 (*C*-2). IR ν_{max} cm^{-1}: 3032 (C-H aromatic), 2823 (C-H aliphatic), 1606 (C=N), 1436 (C=C), 1133 (C-N), 644 (C-S). ESI-MS *m/z* (%): 331 (M^++Na, 22.49%), 309 (M^++H, 13.02%), 218, 204, 186 (100%), 164, 142, 102, 91. Anal. calcd. for $C_{17}H_{16}N_4S$: C, 66.21; H, 5.23; N, 18.17; S, 10.40. Found: C, 66.15; H, 5.48; N, 18.05; S, 10.44.

*6-Phenethyl-2-phenyl-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**1c**)*
Yield 54%, mp 116–117°C. ^{1}H NMR ($CDCl_3$): δ 2.90 (2H; t; -CH_2-Ar), 3.10 (2H; t; -CH_2-CH_2-), 4.71 (2H; s; -S-CH_2), 5.16 (2H; s; -N-CH_2), 7.20–8.06 (10H; m; ArH). ^{13}C-NMR ($CDCl_3$): δ 33.27 (-CH_2-Ph), 51.25 (-N-CH_2-), 60.78 (*C*-5), 67.09 (*C*-7), 128.79 (Ph *C*-4), 126.43 (Ph *C*-2, *C*-6, *C*-4′), 127.85 (Ph *C*-2′, *C*-6′), 128.95 (Ph *C*-3′, *C*-5′), 129.53 (Ph *C*-3, *C*-5), 130.79 (Ph *C*-1), 135.6 (Ph *C*-1′), 151.89 (*C*-3a),

160.70 (*C*-2). IR ν_{max} cm^{-1}: 3029 (C-H aromatic), 2850 (C-H aliphatic), 1602 (C=N), 1438 (C=C), 1134 (C-N), 634 (C-S). ESI-MS *m/z* (%): 345 (M^++Na, 4.73%), 323 (M^++H, 7.10%), 232, 218, 200 (100%), 186, 168, 146, 105. Anal. calcd. for $C_{18}H_{18}N_4S$: C, 67.05; H, 5.63; N, 17.38; S, 9.94. Found: C, 66.74; H, 5.74; N, 17.20; S, 9.90.

*(S)-(−)-2-Phenyl-6-(1-phenethyl)-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**1d**)*
Yield 57%, mp 84–86°C. $[\alpha]_D$ 25° = −56.7 (0.6 mg/mL in $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.48 (3H; d; -CH-C*H*$_3$), 4.07 (1H; q; -C*H*-CH_3), 4.59 (1H; d; *J:* 12.4 Hz; -S-C*H*$_2$), 4.86 (1H; d; *J:* 12.4 Hz; -S-C*H*$_2$), 5.11 (1H; d; *J:* 13.6 Hz; -N-C*H*$_2$), 5.34 (1H; d; *J:* 13.6 Hz; -N-C*H*$_2$), 7.26–8.07 (10H; m; ArH). ^{13}C-NMR ($CDCl_3$): δ 19.20 (*C*H$_3$-), 58.75 (-N-*C*H-Ph), 58.78 (*C*-5), 65.09 (*C*-7), 127.43 (Ph *C*-2, *C*-6), 127.55 (*C*-4′), 128.54 (Ph *C*-2′, *C*-3′, *C*-5′, *C*-6′), 128.79 (Ph *C*-4), 129.30 (Ph *C*-3, *C*-5), 130.79 (Ph *C*-1), 136.6 (Ph *C*-1′), 151.55 (*C*-3a), 160.50 (*C*-2). IR ν_{max} cm^{-1}: 2984 (C-H aromatic), 2848 (C-H aliphatic), 1603 (C=N), 1438 (C=C), 1132 (C-N), 608 (C-S). ESI-MS *m/z* (%): 345 (M^++Na, 4.73%), 323 (M^++H, 15.38%), 232, 219, 200, 190, 156, 148, 105 (100%). Anal. calcd. for $C_{18}H_{18}N_4S$: C, 67.05; H, 5.63; N, 17.38; S, 9.94. Found: C, 66.81; H, 5.53; N, 16.88; S, 9.88.

*2-Benzyl-6-butyl-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**2a**)*
Yield 85%, mp 75–77°C. ^{1}H NMR ($CDCl_3$): δ 0.85 (3H; t; -CH_2-C*H*$_3$), 1.27 (2H; m; -C*H*$_2$-CH_3), 1.46 (2H; m; CH_2-C*H*$_2$-CH_2-), 2.61 (2H; t; -N-C*H*$_2$-CH_2-), 3.86 (2H; s; Ar-C*H*$_2$-), 4.83 (2H; s; -S-C*H*$_2$), 5.09 (2H; s; -N-C*H*$_2$), 7.17–7.27 (5H; m; ArH). ^{13}C-NMR ($CDCl_3$): δ 14.07 (-CH_2-*C*H$_3$), 20.30 (-*C*H$_2$-CH_3), 29.62 (-CH_2-*C*H$_2$-CH_2-), 33.86 (Ph-CH_2-), 50.25 (-N-CH_2-*C*H$_2$-), 55.78 (*C*-5), 67.90 (*C*-7), 125.83 (Ph *C*-4), 128.79 (Ph *C*-3, *C*-5), 129.43 (Ph *C*-2, *C*-6), 136.79 (Ph *C*-1), 150.89 (*C*-3a), 160.70 (*C*-2). IR ν_{max} cm^{-1}: 2930 (C-H aliphatic), 1568 (C=N), 1496 (C=C), 1176 (C-N), 695 (C-S). ESI-MS *m/z* (%): 311 (M^++Na, 31.25%), 289 (M^++H, 90.63%), 258, 252, 236, 216, 184 (100%), 170, 152. Anal. calcd. for $C_{15}H_{20}N_4S$: C, 62.47; H, 6.99; N, 19.43; S, 11.12. Found: C, 62.16; H, 7.09; N, 19.17; S, 11.22.

*2,6-Dibenzyl-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**2b**)*
Yield 82%, mp 130–131°C. ^{1}H NMR ($CDCl_3$): δ 3.93 (2H; s; -N-C*H*$_2$-Ar), 4.04 (2H; s; Ar-C*H*$_2$-), 4.58 (2H; s; -S-C*H*$_2$), 5.11 (2H; s; -N-C*H*$_2$), 7.22–7.38 (10H; m; ArH). ^{13}C-NMR ($CDCl_3$): δ 34.86 (Ph-*C*H$_2$-), 56.25 (-N-CH_2-Ph), 59.78 (*C*-5), 66.89 (*C*-7), 125.79 (Ph *C*-4), 127.43 (Ph *C*-4′), 128.95 (Ph *C*-2, *C*-3, *C*-5, *C*-6, *C*-2′, *C*-3′, *C*-5′, *C*-6′), 135.79 (Ph *C*-1, *C*-1′), 151.49 (*C*-3a), 160.40 (*C*-2). IR ν_{max} cm^{-1}: 3031 (C-H aromatic), 2858 (C-H aliphatic), 1605 (C=N), 1494 (C=C), 1152 (C-N), 698 (C-S). ESI-MS *m/z* (%): 345 (M^++Na, 100%), 323 (M^++H, 84.02%), 226, 218, 204, 186, 120, 91, 60. Anal. calcd. for $C_{18}H_{18}N_4S$: C, 67.05; H, 5.63; N, 17.38; S, 9.94. Found: C, 67.29; H, 5.63; N, 17.21; S, 9.87.

*2-Benzyl-6-phenethyl-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**2c**)*
Yield 81%, mp 78–80°C. ^{1}H NMR (DMSO): δ 2.84 (2H; t; -CH_2-C*H*$_2$-Ar), 2.91 (2H; t; -N-C*H*$_2$-CH_2-), 3.89 (2H; s; Ar-C*H*$_2$-), 4.90 (2H; s; -S-C*H*$_2$), 5.15 (2H; s; -N-C*H*$_2$), 7.19–7.30 (10H; m; ArH). ^{13}C-NMR (DMSO, d_6): δ 32.75 (-CH_2-*C*H$_2$-Ph), 33.86 (Ph-*C*H$_2$-), 50.74 (-N-*C*H$_2$-CH_2-), 54.96 (*C*-5), 67.03 (*C*-7), 125.98 (Ph C-4), 126.11 (Ph C-4′), 128.17 (Ph C-2′, C-6′), 128.54 (Ph C-3, C-5, C-3′, C-5′), 128.64 (Ph C-2, C-6), 138.07 (Ph C-1), 139.20 (Ph C-1′), 146.60 (*C*-3a), 160.33 (*C*-2). IR ν_{max} cm^{-1}: 3027 (C-H aromatic), 2927 (C-H aliphatic), 1604 (C=N), 1440 (C=C), 1120 (C-N), 696 (C-S). ESI-MS m/z (%): 359 (M^++Na, 49.70%), 337 (M^++H, 31.95%), 232 (100%), 200, 146, 134, 105, 71, 60. Anal. calcd. for $C_{19}H_{20}N_4S$: C, 67.83; H, 5.99; N, 16.65; S, 9.53. Found: C, 67.68; H, 6.33; N, 16.48; S, 9.34.

*(S)-6-Butyl-2-[1-(6-methoxy-2-naphthyl)ethyl]-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**3a**)*
Yield 50%, mp 88–90°C. $[\alpha]_D$ 25° = +35.0 (0.6 mg/mL in $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.93 (3H; t; -CH_2-C*H*$_3$), 1.36 (2H; m; -C*H*$_2$-CH_3), 1.53 (2H; m; CH_2-C*H*$_2$-CH_2-), 1.75 (3H; d; -CH-C*H*$_3$), 2.76 (2H; t; -N-C*H*$_2$-CH_2-), 3.89 (3H; s; -OC*H*$_3$), 4.34 (1H; q; -C*H*-CH_3), 4.64 (2H; s; -S-C*H*$_2$), 5.05 (2H; s; -N-C*H*$_2$), 7.09–7.72 (6H; m; ArH). ^{13}C-NMR ($CDCl_3$): δ 14.07 (-CH_2-*C*H$_3$), 20.30 (-*C*H$_2$-CH_3, -CH-*C*H$_3$), 29.62 (-CH_2-*C*H$_2$-CH_2-), 38.86 (-*C*H-CH_3), 50.25 (-N-CH_2-*C*H$_2$-), 55.78 (*C*-5), 55.90 (*C*H$_3$O), 67.90 (*C*-7), 115.83–132.80 (naphthyl *C*-1, *C*-3, *C*-4, *C*-4a, *C*-5, *C*-7, *C*-8, *C*-8a), 133.79 (naphthyl *C*-2), 151.60 (*C*-3a), 156.29 (naphthyl *C*-6), 161.00 (*C*-2). IR ν_{max} cm^{-1}: 2934 (C-H aliphatic), 1606 (C=N), 1440 (C=C), 1264 (C-O), 1031 (C-N), 631 (C-S). ESI-MS *m/z* (%): 405 (M^++Na, 28.40%), 383 (M^++H, 26.04%), 184, 170, 152 (100%), 108, 100, 86, 57. Anal. calcd. for $C_{21}H_{26}N_4OS$: C, 65.94; H, 6.85; N, 14.65; S, 8.38. Found: C, 65.73; H, 6.49; N, 14.28; S, 7.89.

*(S)-6-Benzyl-2-[1-(6-methoxy-2-naphthyl)ethyl]-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**3b**)*
Yield 60%, mp 82–84°C. $[\alpha]_D$ 25° = +47.0 (0.6 mg/mL in $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.77 (3H; d; -CH-C*H*$_3$), 3.90 (3H; s; -OC*H*$_3$), 3.94 (2H; s; -C*H*$_2$-Ar), 4.37 (1H; q; -C*H*-CH_3), 4.56 (2H; s; -S-C*H*$_2$), 5.12 (2H; s; -N-C*H*$_2$), 7.10–7.74 (11H; m; ArH). ^{13}C-NMR ($CDCl_3$): δ 20.30 (-CH-*C*H$_3$), 38.86 (-*C*H-CH_3), 55.90 (*C*H$_3$O), 56.25 (-N-*C*H$_2$-Ph), 59.78 (*C*-5), 67.90 (*C*-7), 115.83–132.80 (naphthyl *C*-1, *C*-3, *C*-4, *C*-4a, *C*-5, *C*-7, *C*-8, *C*-8a, and Ph *C*-2, *C*-3, *C*-4, *C*-5, *C*-6), 133.79 (naphthyl *C*-2), 135.60 (Ph *C*-1), 151.60 (*C*-3a), 156.29 (naphthyl *C*-6), 161.00 (*C*-2). IR ν_{max} cm^{-1}: 2975 (C-H aliphatic), 1606 (C=N), 1455 (C=C), 1264 (C-O), 1030 (C-N), 642 (C-S). ESI-MS *m/z* (%): 439 (M^++Na, 24.26%), 417 (M^++H, 33.73%), 218, 204, 186, 164, 120, 102, 91 (100%). Anal. calcd. for $C_{24}H_{24}N_4OS$: C, 69.20; H, 5.81; N, 13.45; S, 7.70. Found: C, 68.90; H, 6.10; N, 13.32; S, 7.27.

*(S)-6-Phenethyl-2-[1-(6-methoxy-2-naphthyl)ethyl]-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**3c**)*
Yield 83%, mp 138–140°C. $[\alpha]_D$ 25° = +43.0 (0.6 mg/mL in $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.74 (3H; d; -CH-C*H*$_3$), 2.86 (2H; t; -C*H*$_2$-Ar), 3.05 (2H; t; -N-C*H*$_2$-CH_2-), 3.90 (3H; s; -OC*H*$_3$), 4.33 (1H; q; -C*H*-CH_3), 4.62 (2H; s; -S-C*H*$_2$), 5.06 (2H; s; -N-C*H*$_2$), 7.08–7.71 (11H; m; ArH). ^{13}C-NMR ($CDCl_3$): δ 20.30 (-CH-*C*H$_3$), 33.75 (-CH_2-*C*H$_2$-Ph), 38.86 (-*C*H-CH_3), 50.74 (-N-*C*H$_2$-CH_2-), 55.95 (*C*H$_3$O), 60.78 (*C*-5), 67.92 (*C*-7), 118.83–132.80 (naphthyl *C*-1,

C-3, *C*-4, *C*-4a, *C*-5, *C*-7, *C*-8, *C*-8a, and Ph *C*-2, *C*-3, *C*-4, *C*-5, *C*-6), 133.79 (naphthyl *C*-2), 139.60 (Ph *C*-1), 151.60 (*C*-3a), 156.29 (naphthyl *C*-6), 161.00 (*C*-2). IR ν_{max} cm^{-1}: 2846 (C-H aliphatic), 1606 (C=N), 1488 (C=C), 1264 (C-O), 1025 (C-N), 628 (C-S). ESI-MS *m/z* (%): 453 (M^++Na, 15.98%), 431 (M^++H, 20.71%), 232 (100%), 218, 200, 168, 146, 134, 105. Anal. calcd. for $C_{25}H_{26}N_4OS$: C, 69.74; H, 6.09; N, 13.01; S, 7.45. Found: C, 69.66; H, 5.99; N, 12.89; S, 7.24.

*(S,S)-(−)-6-(1-Phenethyl)-2-[1-(6-methoxy-2-naphthyl)-ethyl]-6,7-dihydro-5H-1,2,4-triazolo[3,2-b]-1,3,5-thiadiazine (**3d**)*

Yield 76%, mp 140–142°C. $[\alpha]_D$ 25° = −40.0 (0.6 mg/mL in $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.46 (3H; d; -CH_3-CH-C_6H_5), 1.75 (3H; d; -CH_3-CH-naphtyl), 3.90 (3H; s; -OCH_3), 4.01 (1H; q; -CH-CH_3), 4.35 (1H; q; -CH-CH_3), 4.49 (1H; d; *J:* 12.4 Hz; -S-CH_2), 4.77 (1H; d; *J:* 12.4 Hz; -S-CH_2), 4.99 (1H; d; *J:* 13.6 Hz; -N-CH_2), 5.24 (1H; d; *J:* 13.6 Hz; -N-CH_2), 7.09–7.73 (11H; m; ArH). ^{13}C-NMR ($CDCl_3$): δ 18.80 (CH_3-), 20.50 (-CH-CH_3), 38.86 (-CH-CH_3), 55.95 (OCH_3), 58.78 (*C*-5), 59.75 (-N-CH-Ph), 65.09 (*C*-7), 118.83–132.80 (naphthyl *C*-1, *C*-3, *C*-4, *C*-4a, *C*-5, *C*-7, *C*-8, *C*-8a, and Ph *C*-2, *C*-3, *C*-4, *C*-5, *C*-6), 133.79 (naphthyl *C*-2), 137.60 (Ph *C*-1), 151.60 (*C*-3a), 156.29 (naphthyl *C*-6), 161.00 (*C*-2). IR ν_{max} cm^{-1}: 2976 (C-H aliphatic), 1606 (C=N), 1488 (C=C), 1267 (C-O), 1028 (C-N), 626 (C-S). ESI-MS *m/z* (%): 453 (M^++Na, 28.40%), 431 (M^++H, 30.18%), 327, 232, 218, 200, 172, 156, 105 (100%). Anal. calcd. for $C_{25}H_{26}N_4OS$: C, 69.74; H, 6.09; N, 13.01; S, 7.45. Found: C, 69.67; H, 6.30; N, 12.92; S, 7.14.

Pharmacology

Swiss albino mice of both sexes weighing 20–35 g were used for anti-inflammatory and analgesic activity tests. Mice were housed at a room temperature of 22°C with a 12/12 h light/dark cycle. All animal procedures were performed in accordance with the Institutional Guidelines for Care and Use of Laboratory Animals, and were approved by the Hacettepe University Animal Ethics Committee (2010/33-3). Before starting experimentation, animals were acclimatized to laboratory conditions for 1 week. To eliminate food interference with substance absorption, animals received only water for 12 h prior to experiments. Animals were divided into groups ($n = 6$). Some groups were given a compound (30 mg/kg) in 0.5% carboxymethyl cellulose (CMC) solution intragastrically. The control group received only 0.5% CMC solution. Naproxen (Nap, 30 mg/kg), oxycodone (Oxc, 30 mg/kg), and indomethacin (Indo, 10 mg/kg) were chosen as the reference drugs.

Analgesic activity evaluation in animal models: Hot plate test

For determination of antinociceptive activity, a conventional hot plate test was performed according to the methods described by Eddy and Leimbach [16]. Mice ($n = 6$) were placed individually on a hot plate (9601 Analgesic Hot Plate, Commat Ltd., Ankara) set at 50 ± 0.5°C and a plexiglass cylinder (12 cm diameter, 20 cm height) was used to restrict the mouse on the heated surface of the plate. The time elapsed until the start of paw licking or a jump response (whichever appeared first) was recorded as an index of nociception. Only mice that showed a nociceptive response within 15 s were used in the experiment. The maximum cut-off time was chosen as 15 s to prevent tissue damage. After baseline readings (T_0) were taken, the mice were treated with test compounds or references. Response latencies (T_1) were measured 2 h after drug application. The effects of the compounds on nociception were determined via the percentage of maximal possible effect (MPE%), which was calculated as below:

$$\text{MPE}\ (\%) = (T_1 - T_0)/(T_2 - T_0) \times 100$$

where the cut-off time (T_2) was 15 s.

Analgesic activity evaluation in animal models: Tail-flick test

For the tail-flick test, an automated tail-flick apparatus (TF 0703, 8 V/50 W Tail-flick, Commat Ltd., Ankara) was used, which elicited a response by applying radiant heat to the dorsal surface of the tail. For the experiment, each mouse was gently held and an automatic timer measured latency until the mouse flicked its tail away from the source of the light. The heat stimulus was set to provide a pre-drug tail-flick response time of 6–8 s. The cut-off time for the heat stimulus was set at 15 s to prevent tissue damage [17]. After baseline readings (T_0) were taken, the mice were treated with test compounds or references. Response latencies (T_1) were measured 2 h after application. The analgesic activity was calculated as the percentage maximum possible effect (MPE%) using the following formula:

$$\text{MPE}\ (\%) = (T_1 - T_0)/(T_2 - T_0) \times 100$$

where the cut-off time (T_2) was 15 s.

Anti-inflammatory activity evaluation in animal models: Carrageenan-induced paw edema test

The anti-inflammatory properties of the synthesized compounds were examined through a modified carrageenan induced paw edema test previously described by Winter et al. [18]. Edema was stimulated by the injection of 0.01 mL 2% carrageenan suspension into the subplantar region of the right hind paw with a Hamilton injector. Carrageenan was injected 1 h after gavage. Paw thickness was measured using a dial thickness gauge (0.01–1 mm, Ozaki Co., Japan) immediately before (T_0) and 2 h (T_t) after injection. Edema was calculated as the increase in paw thickness (mm) after treatment subtracted from the basal volume ($\Delta T = T_t - T_0$). For each animal, edema inhibition was expressed as the percentage of their control, with the formula shown below:

$$\text{Anti-Inflammatory Activity}\ (\%) = [(\text{Control}\ \Delta T - \text{Test}\ \Delta T)/\text{Control}\ \Delta T] \times 100$$

Ulcerogenic liability

Ulcerogenic activity was investigated after the carrageenan-induced paw edema test. All groups were starved for 12 h, but water was provided ad libitum. Two hours following the final

dose, mice were euthanized. The stomach of each mouse was removed, opened along the greater curvature, rinsed with 0.9% sodium chloride (isotonic solution), and stretched by using pins on a cork board. The lesions in the gastric mucosa were determined using a dissecting microscope. The quantification of gastric mucosal lesions was scored according to their number and size on a scale from 0 to 7 points, adapted from Magistretti et al. [19] as follows:

(0) without injury,
(1) color modification,
(2) few petechia/alterations of villous,
(3) 1–3 small injuries ($\leq$1 mm length),
(4) 1–3 large injuries ($\geq$1 mm length),
(5) $>$4 large injuries ($>$1 mm),
(6) Several continuous lined injuries or small ulcers without bleeding,
(7) Several massive bleeding or digested blood in stomach.

Acute toxicity study

The animals were observed continuously for 6 h after treatment, then intermittently for 72 h, and thereafter, for over a period of 14 days after administration [20] for behavioral changes, signs of toxicity, and/or death.

Statistical analysis

All data are expressed as mean $\pm$ standard error of mean (SEM). Results of carrageenan-induced paw edema experiments are also expressed as a percentage of change from control (pre-drug) values. Differences between vehicle control, reference drugs, and treatment groups were tested using a one-way analysis of variance (ANOVA) followed by a *post-hoc* Dunnett's test. Ulcer scores were analyzed using the Kruskal–Wallis test followed by a *post-hoc* Dunn's test.

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The authors declare that they have no conflict of interest.

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