Biological Activity of Novel N-Substituted Amides of *endo*-3-(3-Methylthio-1,2,4-triazol-5-yl)bicyclo[2.2.1]hept-5-ene-2-carboxylic Acid and N-Substituted Amides of 1-(5-Methylthio-1,2,4-triazol-3-yl)cyclohexane-2-carboxylic Acids

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N-Substituted amides of *endo*-3-(3-methylthio-1,2,4-triazol-5-yl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid and 1-(5-methylthio-1,2,4-triazol-3-yl)cyclohexane-2-carboxylic acid were prepared by the condensation reaction of *endo*-*S*-methyl-N^1-(bicyclo[2.2.1]hept-5-ene-2,3-dicarbonyl)isothiosemicarbazide and *S*-methyl-N^1-(cyclohexane-2,3-dicarbonyl)isothiosemicarbazide with primary amines. The synthesized compounds were screened for their microbiological and pharmacological activities.

Key words: 1,2,4-Triazole, Bioactivity

Introduction

During recent years an increasing number of cases of bacterial diseases caused by strains resistant to antimicrobials, *e.g.* antibiotics, has been noted (Sriram *et al.*, 2005; Narain *et al.*, 2002; Sbarbaro, 1997). One of the most important points in modern medicine is to pay attention to the progress in novel approaches to antimicrobial therapy (Capobianco *et al.*, 2000; Chopra, 2001; Choudhry *et al.*, 2003; Oliva *et al.*, 2003).

Five-membered heterocyclic compounds containing nitrogen, especially 1,2,4-triazole and its derivatives, are highly prevalent in collections of bioactive compounds. It is well-known that they can be applied as anti-inflammatory (Amir and Shikha, 2004), antidepressant (Chiu and Huskey, 1998), antiviral (Al-Soud *et al.*, 2004), anticonvulsant (Almasirad *et al.*, 2004), analgesic (Kumar *et al.*, 2008), and anticancer (Demirbas and Ugurluoglu, 2002) agents, respectively. Moreover, some of them possess antifungal (Turan-Zitouni *et al.*, 2005), antimicrobial (Demirbas *et al.*, 2005), and antitubercular (Walczak *et al.*, 2004) properties. Besides, a literature survey reveals that the 1,2,4-triazole system is the structural nucleus of vorozole, letrazole, and anastrozole (Clemons *et al.*, 2004; Goss and Strasser-Weippl, 2004; Santen, 2003) which are very effective nonsteroidal aromatase inhibitors. Virazole (Shigeta, 2000) is an antiviral agent. Alprazolam (De Witte *et al.*, 2002) is used as an anxiolytic agent, nefazodone (Spina *et al.*, 2008) and trazodone (Kast, 2009) are antidepressant drugs, fluconazole (Tsukuda *at al.*, 1998) and intraconazole (Bailey *et al.*, 1990) are used as antifungal drugs. Therefore triazoles are potential scaffolds for the design of bioactive compounds.

Prompted by these reports, and in continuation of our search for bioactive molecules (Pitucha *et al.*, 2009, 2010), it seemed worthwhile to synthesize derivatives of 1,2,4-triazole, containing the amide and methylthio group like compounds with potential biological activity (Pachuta-Stec *et al.*, 2009). The syntheses and molecular structures have been described earlier (Mendyk *et al.*, 2011; Galewicz-Walesa and Pachuta-Stec, 2003). Here we report the evaluation of the results of the preliminary microbiological investigation. Two of the synthesized compounds, **4a** and **10a**, were also examined with reference to their effect on the central nervous system (CNS) of mice. These compounds were chosen as prototypes for the ali-

phatic and aromatic series of side chains. Moreover, triazoles with chlorophenyl and butyl substituents attached to the heterocyclic system had been shown to exhibit moderate to good CNS activity in mice (Amir *et al*., 2008; Kane *et al*., 1990; Karthikeyan, 2009; Tozkoparan *et al*., 2007).

Results and Discussion

The starting *endo-S*-methyl-N^1-(bicyclo[2.2.1]-hept-5-ene-2,3-dicarbonyl)isothiosemicarbazide and *S*-methyl-N^1-(cyclohexane-2,3-dicarbonyl)-isothiosemicarbazide, used in this study, were obtained by the direct condensation of *S*-methyl isothiosemicarbazide hydroiodide with a suitable dicarboxylic acid anhydride. The N-substituted amides were prepared by the condensation of the abovementioned isothiosemicarbazides with aliphatic and aromatic primary amines in boiling glacial acetic acid (Mendyk *et al.*, 2011; Galewicz-Walesa and Pachuta-Stec, 2003). The chemical structures of the obtained and examined compounds are presented in Fig. 1.

According to our preliminary results based on the agar well diffusion method, among the tested agents **1a**–**13a** and **1b**–**12b**, only compounds **1a**, **7a**, **7b**, **9b**, and **13a** showed potential activity against Gram-positive or Gram-negative bacteria, as monitored by the growth inhibition zone around the well with the diameter ranging from 13 to 16 mm. On the basis of the MIC (minimal inhibitory concentration) values obtained by the broth microdilution method, it became clear that only few of these compounds had moderate ac-

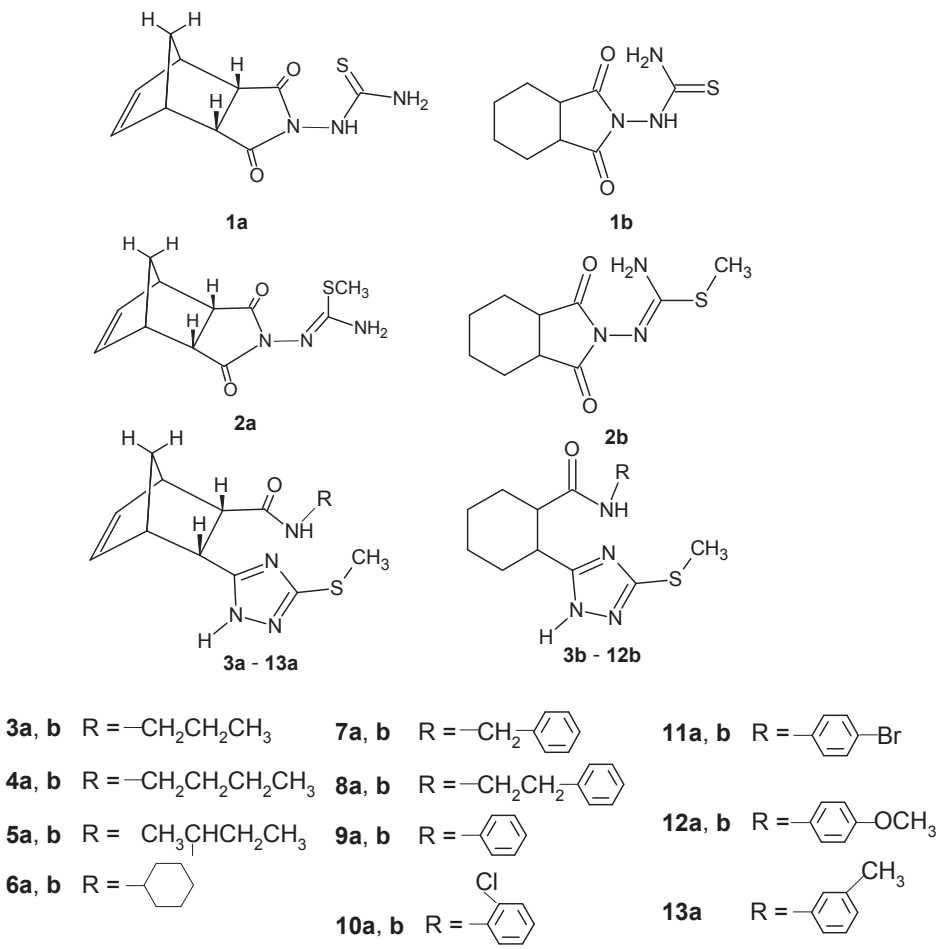

Fig. 1. Chemical structures of compounds **1a**, **1b**–**12a**, **12b** and **13a**.

tivity against Gram-positive bacteria. The compounds **7b**, **9b**, and **13a** were active against *Staphylococcus aureus* ATCC 25923 (MIC = 500 μg/mL), compounds **1a**, **7b**, and **13a** against *S. epidermidis* ATCC 12228 (MIC = 500 μg/mL), while **7a** was active against *Micrococcus luteus* ATCC 10240 (MIC = 250 μg/mL). Besides, compound **7a** also exhibited activity against the Gram-negative *Escherichia coli* ATCC 25922 (MIC = 500 μg/mL). In our experiments, MIC values for available antibiotics, such as cefuroxime, which has been extensively used for treating bacterial infections, were also estimated; they were 0.24 to 1.95 μg/mL for *Staphylococcus* species and 0.49 to 31.25 μg/mL for the other Gram-positive bacteria.

Among the tested compounds only **11a** affected the growth of fungi belonging to yeasts (*Candida albicans* ATCC 10231, *C. albicans* ATCC 2091, *C. parapsilosis* ATCC 22019) with partial growth inhibition around the wells and MIC values ranging from 250 to 500 μg/mL.

Summing up, among the tested agents, only compounds **1a**, **7a**, **7b**, **9b**, and **13a** may be valuable for searching new derivatives showing better antimicrobial activity against bacteria (*e.g. S. aureus*, *S. epidermidis*, *M. luteus*, or *E. coli*) or yeasts (*e.g. C. albicans* or *C. parapsilosis*).

Preliminary pharmacological results have shown that compounds **4a** and **10a** do not display neurotoxic activity and weakly affect the mice CNS. Both compounds had analgesic activity in the writhing syndrome test (Table I). Compound **10a**, *N*-(2-chlorophenyl) amide of *endo*-3-(3-methylthio-1,2,4-triazol-5-yl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, was more active because in a wide range of doses it decreased the pain reactivity of mice in this test. The analgesic effect of **10a** is interesting and should be further examined in more detail. In the other behavioural tests, the investigated N-substituted amides of *endo*-3-(3-methylthio-1,2,4-triazol-5-yl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid had basically no effect. The hole board test and the four plate test did not reveal any potential anxiolytic character of the tested compounds. The forced swimming test did not show antidepressant effects on the behaviour of mice. The investigated compounds did not prolong thiopental sleeping time and did not show any anticonvulsant effect. In the head twitch test, none of the compounds showed antiserotoninergic activity. Summing up, it was shown that compound **10a** has analgesic activity which will be further investigated and tested on rodents.

Table I. Antinociceptive activity of **4a** and **10a** in the writhing syndrome test in mice (N = 8).

Compound	Treatment (mg/kg i.p.)	Mean writhing number	Inhibition[a] (%)
Control	–	63.0 ± 7.2	0
4a	50.0	49.0 ± 3.6	14.7
	100.0	32.3 ± 4.6**	48.7**
Control	–	62.6 ± 3.9	0
10a	12.5	53.3 ± 5.8	14.8
	25.0	35.9 ± 9.6**	42.6**
	50.0	9.4 ± 4.5**	84.9**
	100.0	7.9 ± 2.9**	87.4**

[a] Percent of inhibition obtained by comparison with the control group.
**$p < 0.001$ vs. the control group.

Experimental

Microbiology

The new compounds **1a**–**13a** and **1b**–**12b** were screened for their antimicrobial activity *in vitro* against eight species of aerobic bacteria and six species of fungi. All these microorganisms came from the American Type Culture Collection (ATCC; Dziekanów Leśny, Poland) and are routinely used for evaluation of antimicrobials. The reference strains of aerobic bacteria included Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 10240) and Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 9027), as well as fungi (*Candida albicans* ATCC 10231, *C. albicans* ATCC 2091, *C. parapsilosis* ATCC 22019, *Trichophyton menthagrophytes* ATCC 9533, *Aspergillus niger* ATCC 16404).

Microbial suspensions were prepared in sterile saline (0.85% NaCl) with an optical density of 0.5 McFarland standard, $150 \cdot 10^6$ CFU (colony forming units)/mL. All stock solutions of the tested compounds were dissolved in dimethyl sulfoxide (DMSO). It was found that DMSO at the final concentration employed did not affect the growth of the tested microorganisms.

Antimicrobial activities of the newly synthesized compounds were screened by the agar well

diffusion method and, for potentially active ones, by the broth microdilution technique. Using the agar well diffusion method, the antimicrobial activity of the tested agents was expressed as the average diameter of the growth inhibition zone surrounding the well containing the compounds at a concentration of 1000 μg/mL. Mueller-Hinton agar or Mueller-Hinton agar supplemented with 2% glucose were used. Sterile swabs were used to spread the microbial suspensions onto the medium surface, and then the solution of each compound was introduced into the wells (80 μL per each well). The wells (d = 8 mm) were made in the agar with a sterile cork-borer. The plates were preincubated at room temperature for 1.5 h to allow the diffusion of solution into the medium, and then they were incubated at 37 °C for 18 h (for bacteria) or at 30 °C for 48 h (for fungi).

Further experiments were focused on the assessment of the activity of compounds **1a**, **7a**, **7b**, **9b**, **11a**, and **13a** allowing for MIC determination using the broth microdilution method (OD_{600}). Mueller-Hinton broth (for bacteria) or Mueller-Hinton broth with 2% glucose (for fungi) containing compounds at concentrations of 31.25–500 μg/mL were used. This technique was developed using 96-well microplates which were inoculated with an 1:10 diluted microbial suspension of an optical density of 0.5 McFarland standard; 20 μL of bacterial suspension were transferred into 180 μL of medium containing a two-fold dilution of the tested compounds. After incubation (at 37 °C for 18 h for bacteria and at 30 °C for 24 h for fungi), the optical densities (OD_{600}) were determined for bacterial cultures in the broth medium and the MIC values were determined by comparison with the growth of the control (compound-free) medium. Cefuroxime, belonging to the 2nd generation of cephalosporins, was used as a control antimicrobial agent at final concentrations of 0.063 to 500 μg/mL.

Pharmacological studies

The experiments were carried out on male Albino Swiss mice (20–24 g). The animals were kept in colony cages with free access to food (standard laboratory pellets; Bacutil Motycz, Poland) and tap water, and maintained in the natural light-dark cycle. The experiments were performed between 8 a.m. and 2 p.m. The investigated compounds **4a** and **10a** were administered intraperitoneally (i.p.) in doses of 12.5, 25, 50, and 100 mg/kg body, respectively, as suspensions in 1% aqueous Tween 80 solution at a constant volume of 0.1 mL per 10 g body weight of mice. Control animals received an equivalent volume of the solvent. The tested groups consisted of 8 mice. The Bioethical Committee of Lublin Medical University had approved all experimental procedures applied in this study.

Screening of the CNS activity in mice was performed in a series of tests described below. Motor coordination was quantified with the chimney test (Boissier *et al.*, 1960). The rectal body temperature in mice was measured by an Ellab thermometer. The effects on the explorative activity were determined applying the hole board test (Boissier and Simon, 1967). Anxiolytic activity was assessed by the four plate test in mice according to Aron *et al.* (1971). The passive avoidance task, which is considered to be a measure of long-term memory in rodents, followed the procedure of Venault *et al.* (1986). Antidepressant properties were assessed by the forced swimming test (Porsolt *et al.*, 1977). Thiopental-induced sleep was measured too. Analgesic activity was measured by the writhing syndrome test (Witkin *et al.*, 1961). Antiepileptic effects were tested by reduction of pentetrazole (100 mg/kg body weight)-induced seizures. Antiserotoninergic effects were determined by the Corne test (Corne *et al.*, 1963).

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