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Application of *thyme* essential oil for the synthesis and *in silico* analysis of novel 2-substituted benzimidazolines

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The wide range of biological properties possessed by essential oils containing phenolic monoterpenoids makes them appealing for the development of new biologically active compounds. Thymol is the primary phenolic monoterpenoid found in essential oils of *Thymus vulgaris*. As an addition to its bioactive profile, thymol is a highly promising scaffold for future synthetic modifications toward new hybrid molecules with potential biological activities.

The present study aims at the regioselective *α*-amidoalkylation of thymol and thyme essential oil, thereby expanding the applicability of this synthetic approach. The reactions were carried out by *in situ* generated *N,N*-diacyliminium reagents formed from benzimidazole or 5,6-dimethylbenzimidazole with methyl or ethyl chloroformate in dichloromethane at room temperature. Four novel *N,N*-diacylated benzimidazoline-thymol hybrids were synthesized (4a-d) with yields in the range from 65% to 83%. The conducted *in silico* toxicity analysis revealed that the compound (4a) exhibited the lowest predicted toxicity (1298.76 mg/kg) among the other synthesized analogues. The structure of the synthesized compounds was characterized using ^{1}H-, ^{13}C-NMR, FTIR spectroscopic analyses and HRMS data.

Keywords: *α*-amidoalkylation, benzimidazole, monoterpenoid hybrids, thymol, thyme essential oil, *in silico* analysis

INTRODUCTION

The synthesis of novel 2-substituted benzimidazoles with a broad spectrum of biological activities such as antibacterial [1–3], antiproliferative [1, 4, 5], anticancer [2, 5], anthelmintic [2, 3, 5], antiviral [6, 7], antioxidant [8–10], photoprotective [8, 9], antispasmodic [11], among others, is crucial for the discovery of potentially active pharmacological agents [12]. Modern bioactive molecules design often involves the development of novel hybrid molecules [13]. Molecular hybridization is a strategic approach in medicinal chemistry aimed at the synthesis of novel molecules with an improved biological activity and therapeutic potential [13, 14]. *α*-Amidoalkylation reactions are important in organic synthesis for constructing a carbon–carbon (C-C) bond [15] and have found widespread application in the design and synthesis of molecular hybrids [16, 17]. Natural products, including components of essential oils such as thymol, known for its antibacterial [18], anti-inflammatory [19], antioxidant [20], and other properties, represent an important source for the development of novel bioactive compounds [21].

In a recently published study, we applied thyme and oregano essential oils for molecular hybridization. We successfully combined benzothiazole and natural monoterpenoids to obtain new bioactive compounds with promising UV-B and antimicrobial activity. As addition, a low toxicity was established through *in silico* analysis, and SPF values are comparable to the commercial filter PBSA [22]. In this context, a similar strategy could be applied for the synthesis of new benzimidazole derivatives containing a thymol fragment.

The synthetic group of Itoh *et al*. [23, 24] synthesized 2-substituted compounds by activation of the benzimidazole ring with ethyl chloroformate. Such types of syntheses are applied in the development of bioactive molecules. For the first time, an adduct of benzimidazole and ethyl chloroformate was isolated and spectrally characterized by Venkov and Statkova [25]. The authors established that the adduct is sufficiently stable to be isolated. Later, the same authors synthesized a series of 1,2,3-trisubstituted 2-(2-oxoalkyl)-2,3-dihydrobenzoimidazoles and investigated their antiproliferative activity. One of the products obtained via amidoalkylation of benzalacetone (Figure 1), exhibited pronounced selective antiproliferative activity against human metastatic melanoma (A2058) at a concentration of 10^{-4} M [4].

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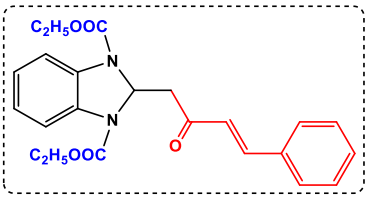


Figure 1. Benzimidazoline-benzalacetone hybrid.

The literature data and the presented results provide a solid basis for conducting experimental studies targeted to the synthesis and evaluation of new compounds that combine a benzimidazole core with another natural fragment. In this regard, the aim of the current study was the pplication of thyme essential oil for the synthesis of novel benzimidazoline-thymol hybrids by reaction of *α*-amidoalkylation and their subsequent *in silico* analysis.

EXPERIMENTAL

General information

Commercial solvents and reagents, such as benzimidazole, 5,6-dimethylbenzimidazole, alkyl chloroformates, thymol and thyme essential oil were purchased from Sigma-Aldrich (Merck EAD, Sofia, Bulgaria). Melting points were measured on a Boetius PHMKO5 hot-stage apparatus (Carl Zeiss Jena, Germany). FTIR spectra were taken in KBr tablets on a Perkin Elmer 1750 Fourier Transform spectrometer. For HRMS analysis a HRMS Q-Exactive Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) spectrometer was successfully applied. ^{1}H-, ^{13}C-NMR spectra were recorded on Bruker Avance AV600 spectrometer (Bruker, Billerica, MA, USA) at IOCCP-BAS, Sofia, in DMSO-d_6 as a solvent. To average out the rotamers reaching adequate assignment of peaks and structure determination, the spectra were measured at 80 °C. TLC was done on precoated 0.2 mm Merck silica gel 60 plates with eluent mixture of petroleum ether:diethyl ether 2:1. All pure samples were isolated by recrystallization from petroleum ether:diethyl ether in various ratios described in the general procedures.

General procedure for amidoalkylation of thymol using adducts derived from benzimidazole or 5,6-dimethylbenzimidazole with alkyl chloroformate

To a solution of 1 mmol benzimidazole or 5,6-dimethylbenzimidazole in 10 ml dichloromethane, 1 mmol of triethylamine (Et_3N) is added as a hydrogen chloride (HCl) scavenger, followed by slow dropwise addition of the acid chloride – either 2.4 mmol ethyl chloroformate or 2.8 mmol methyl chloroformate. Immediately afterward, 1 mmol of thymol is added. The reaction mixture is stirred with a magnetic stirrer for 30 min at 0 °C, then left at room temperature for 24 h. The product is isolated by extraction with 3×20 ml of CH_2Cl_2, sequentially with 30 ml of hydrochloric acid:water (1:4) solution and 50 ml of water. The resulting organic extract is dried over anhydrous sodium sulfate (Na_2SO_4), and the solvent is evaporated. The products (4a-d) are isolated as white powders by recrystallization from petroleum ether:diethyl ether 4:1 with increasing polarity to 1:1.

General procedure for the synthesis of 2-substituted benzimidazoles by efficient One-pot reaction with thyme essential oil

To benzimidazole or 5,6-dimethylbenzimidazole (2 mmol) dissolved in 10 ml of dry dichloromethane, 2 mmol of triethylamine (Et_3N), alkyl chloroformate – 2.5 mmol ethyl chloroformate or 3 mmol methyl chloroformate are successively added and the reaction mixture is left for 20 min in an ice bath (-4–0 °C). After 20 min a second equivalent of alkyl chloroformate is added to the reaction mixture, and adduct formation follows at room temperature 25 ºC for 20 min. The third stage of the reaction proceeds successfully without the need of isolating the acyliminium reagents by adding 1.2 g (containing at least 2.9 mmol of thymol) of essential oil to the same reaction vessel. Then, the reactions proceed for 24 h at room temperature. The products (4a-d) are isolated according to the abovementioned successful procedure by recrystallization from petroleum ether:diethyl ether 8:1 with increasing polarity to 4:1. The obtained yields are calculated based on benzimidazole. In these experiments, it was found that the acyliminium reagents were stable in the presence of the essential oil over the course of 24 h.

Spectral characterization of the obtained compounds

Dimethyl 2-(4-hydroxy-5-isopropyl-2-methylphenyl)-1H-benzo[d]imidazole-1,3(2H)-dicarboxylate (4a); Yield: (65 %); Melting point: 200–202 ºC; ^{1}H-NMR (600 MHz, 80 ºC DMSO-d_6, δ ppm, J Hz): 1.15 (d, J=7.04 Hz, 6 H), 2.50 (s, 3 H), 3.18-3.23 (m, 1 H), 3.86 (s, 6 H), 6.73 (s, 1 H), 7.02 (s, 1 H), 7.13 (s, 1 H, CH), 7.22-7.25 (m, 2 H), 7.78 (br. s., 2 H), 9.05 (br. s., 1 H, OH); ^{13}C-NMR (150 MHz, 80 ºC, DMSO-d_6, δ ppm, J Hz) 18.2, 22.7, 26.7, 53.2, 74.6, 114.1, 117.2, 123.7, 124.00, 128.8, 132.7, 134.6, 151.8, 155.1; FTIR (KBr, cm^{-1}): 3426, 3364, 2956, 1716, 1690, 1622, 1596, 1503, 1445, 1385, 1313, 1280, 1197, 1136, 1025, 958, 859, 827, 749, 572; HRMS *m/z* (ESI): calcd for $C_{21}H_{24}N_2NaO_5^+$ $[M+Na]^+$ 407.1577, found

407.1579; calcd for $C_{21}H_{23}N_2O_5^-$ $[M-H]^-$ 383.1612, found 383.1610;

Diethyl 2-(4-hydroxy-5-isopropyl-2-methylphenyl)-1H-benzo[d]imidazole-1,3(2H)-dicarboxylate (4b); Yield: (83 %); Melting point: 191–193 ºC; ^{1}H-NMR (600 MHz, 80 ºC DMSO-d_6, δ ppm, J Hz): 1.19 (d, J=7.04 Hz, 6 H), 1.32 (t, J=6.46 Hz, 6 H), 2.50 (s, 3 H), 3.22-3.26 (m, 1 H), 4.32 (q, J=7.04 Hz, 4 H), 6.75 (s, 1 H), 7.07 (s, 1 H, CH), 7.13 (s, 1 H, CH), 7.25 (d, J=2.93 Hz, 2 H), 7.84 (br. s., 2 H), 9.19 (br. s., 1 H, OH); ^{13}C-NMR (150 MHz, 80 ºC, DMSO-d_6, δ ppm, J Hz) 14.4, 18.3, 22.7, 26.8, 62.1, 75.0, 114.0, 117.1, 123.6, 124.5, 128.9, 132.6, 132.9, 134.6, 151.3, 155.0; FTIR (KBr, cm^{-1}): 3405, 2966, 1719, 1678, 1621, 1590, 1501, 1409, 1378, 1378, 1281, 1275, 1190, 1035, 871, 851, 759, 740, 576; HRMS *m/z* (ESI): calcd for $C_{23}H_{28}N_2NaO_5^+$ $[M+Na]^+$ 435.1890, found 435.1887; calcd for $C_{23}H_{27}N_2O_5^-$ $[M-H]^-$ 411.1925, found 411.1928;

Dimethyl 2-(4-hydroxy-5-isopropyl-2-methylphenyl)-5,6-dimethyl-1H-benzo-[d]imidazole-1,3(2H)-dicarboxylate (4c); Yield: (73 %); Melting point: 215–216 ºC; ^{1}H-NMR (600 MHz, 80 ºC DMSO-d_6, δ ppm, J Hz): 1.20 (d, J=6.46 Hz, 6 H), 2.44 (s, 6 H), 2.50 (s, 3 H), 3.21-3.25 (m, 1H), 3.86 (s, 6 H), 6.74 (s, 1 H), 7.04 (s, 1 H), 7.08 (s, 1 H, CH), 7.60 (br. s., 2 H), 9.27 (br. s., 1 H, OH); ^{13}C-NMR (150 MHz, 80 ºC, DMSO-d_6, δ ppm, J Hz) 18.2, 20.5, 22.7, 26.9, 52.3, 74.8, 115.3, 117.1, 124.3, 128.8, 131.5, 132.6, 134.6, 143.4, 151.7, 155.0; FTIR (KBr, cm^{-1}): 3417, 3191, 2957, 2930, 2868, 1722, 1699, 1514, 1447, 1384, 1198, 1124, 984, 869, 755, 570; HRMS *m/z* (ESI): calcd for $C_{23}H_{28}N_2NaO_5^+$ $[M+Na]^+$ 435.1890, found 435.1892; calcd for $C_{23}H_{27}N_2O_5^-$ $[M-H]^-$ 411.1925, found 411.1923;

Diethyl 2-(4-hydroxy-5-isopropyl-2-methylphenyl)-5,6-dimethyl-1H-benzo-[d]imidazole-1,3(2H)-dicarboxylate (4d); Yield: (68 %); Melting point: 203–206 ºC; ^{1}H-NMR (600 MHz, 80 ºC DMSO-d_6, δ ppm, J Hz): 1.24 (d, J=6.46 Hz, 6 H), 1.33 (t, J=6.75 Hz, 6 H), 2.46 (s, 6 H), 2.50 (s, 3 H), 3.25-3.29 (m, 1H), 4.32 (q, J=7.04 Hz, 4 H), 6.77 (s, 1 H), 7.09 (d, J=2.93 Hz, 2 H), 7.67 (br. s., 2 H), 9.22 (br. s., 1 H, OH); ^{13}C-NMR (150 MHz, 80 ºC, DMSO-d_6, δ ppm, J Hz) 14.4, 18.3, 19.8, 22.7, 26.9, 61.9, 75.2, 115.2, 117.1, 124.8, 129.0, 130.8, 132.5, 134.6, 151.2, 155.0; FTIR (KBr, cm^{-1}): 3408, 2970, 2930, 2868, 1716, 1678, 1514, 1407, 1289, 1127, 1018, 879, 759, 575; HRMS *m/z* (ESI): calcd for $C_{25}H_{32}N_2NaO_5^+$ $[M+Na]^+$ 463.2203, found 463.2205; calcd for $C_{25}H_{31}N_2O_5^-$ $[M-H]^-$ 439.2238, found 439.2241.

In silico T.E.S.T. analysis

To predict selected properties using *in silico* methods, computational analysis was applied in this study using freely available software - T.E.S.T. (version 5.1.2) [28]. Table 3 presents data on the properties of the newly synthesized compounds (4a-d). The T.E.S.T. software, developed by the U.S. Environmental Protection Agency (EPA, Washington, DC), was used to evaluate oral rat LD_{50} (mg/kg), mutagenicity and water solubility (mg/l) at 25 °C, employing the nearest neighbour machine learning method. The toxicological profile and physicochemical properties of the newly synthesized compounds were assessed. The obtained results are presented in Table 3.

RESULTS AND DISCUSSION

The present study applies the concept of molecular hybridization by combining a benzimidazole fragment with a natural, biologically active monoterpenoid phenol - thymol. As the main synthetic approach, a multicomponent α-amidoalkylation reaction was employed for the synthesis of the target *N*,*N*-diacylated benzimidazoline–thymol hybrids (4a-d) (Scheme 1).

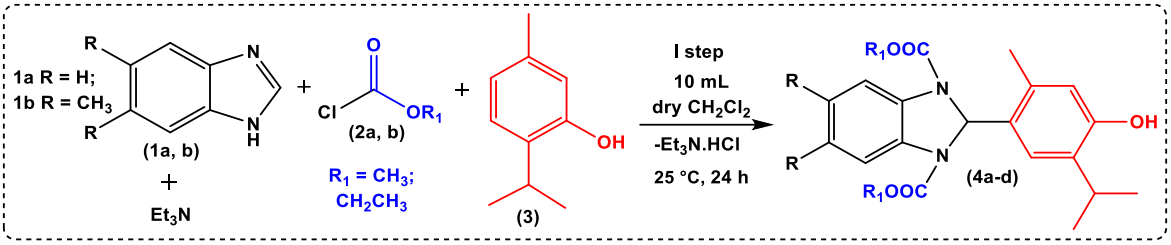
The reaction conditions and yields of thymol-containing hybrid molecules are presented in Table 1.

Table 1. Yields of thymol hybrids (4a-d) obtained according to Scheme 1.

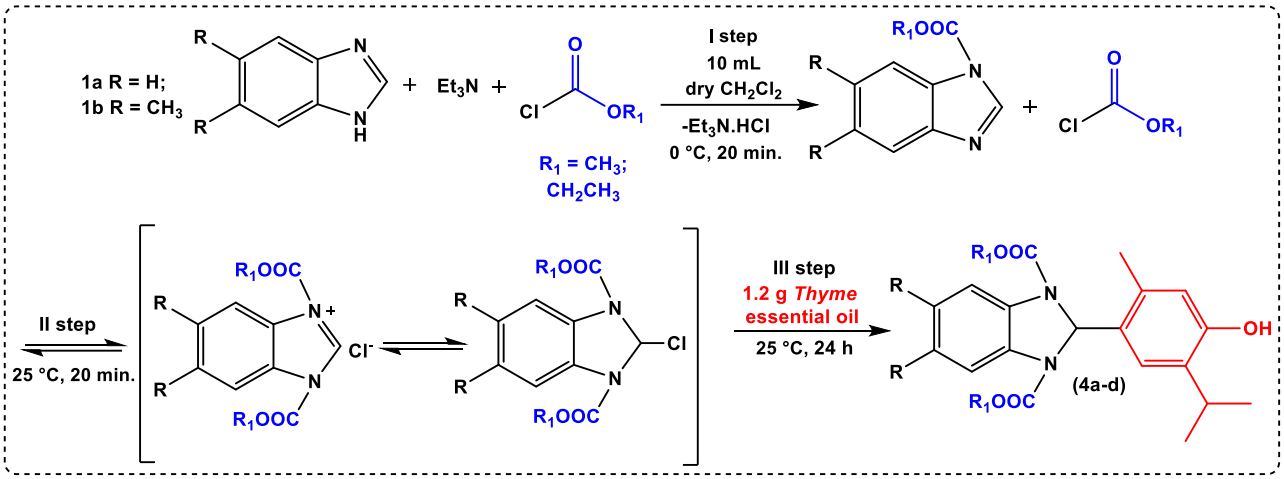
Compound (4)	R	R_1	Yield, %
a	H	Me	65
b	H	Et	83
c	CH_3	Me	73
d	CH_3	Et	68

The obtained experimental data indicate that under these reaction conditions, the substitution predominantly occurs at the *para*-position of the aromatic ring. The observed regioselectivity of the amidoalkylation reaction provides a rationale approach for extending the study by applying the same synthetic approach to more complex natural matrices, such as essential oils.

Building on previous work and the recently published study on the amidoalkylation of thyme essential oil (thymol chemotype) with acyliminium reagents derived from benzothiazole and alkyl chlorformates [22], the investigations were extended using benzimidazole, 5,6-dimethylbenzothiazole, methyl, and ethyl chloroformates to obtain new hybrid molecules.



Scheme 1. *α*-Amidoalkylation reaction for the synthesis of thymol hybrids (4a-d).



Scheme 2. Application of thyme essential oil in *α*-amidoalkylation reaction for the synthesis of thymol hybrids (4a-d).

In the present study, one-pot synthetic strategy was successfully applied, in which all reaction steps proceeded sequentially in a single reaction vessel without the need for isolation and purification of the intermediates. The key steps of the synthetic pathway, including the used reagents and the reaction conditions, are presented in Scheme 2. Essential oil (EO) was purchased from a commercial source, with thyme oil characterized by the 'Standard Winter' chemotype. According to the corresponding ISO specifications, this chemotype should contain thymol within the range of 35% to 55% [26]. To verify the chemical composition of the commercial samples, gas chromatography–tandem mass spectrometry (GC-MS/MS) was conducted in accordance with a validated analytical method [27]. The results confirmed that the composition of the analysed EO met the criteria outlined in the applicable ISO standard. The amount of thyme oil used was deliberately selected (1.2 g) to correspond to the lowest permissible thymol content (35%) according to the ISO standard 19817:2017 [26]. Under these reaction conditions, the amidoalkylation reaction proceeded successfully. The reaction progress and the formation of the target products were monitored by thin-layer chromatography (TLC), using the corresponding reference compounds (4a-d) for comparative analysis. The target compounds synthesized using thyme essential oil, were isolated by recrystallization with yields ranging from 66% to 70% and are presented in Table 2.

Table 2. Yields of thymol hybrids (4a-d) obtained from thyme essential oil according to Scheme 2.

Compounds (4)	R	R_1	Yield, %
a	H	Me	69
b	H	Et	70
c	CH_3	Me	66
d	CH_3	Et	67

As a result of the conducted syntheses, four novel crystalline compounds were obtained with good yields ranging from 65% to 83% (Tables 1, 2). They were isolated by recrystallization. The synthesized compounds were characterized using ^{1}H- and ^{13}C-NMR, FTIR spectroscopy, and high-resolution mass spectrometry (HRMS), which unequivocally confirmed their structures.

In comparison with the previously obtained benzothiazole–thymol hybrid molecules [22], the adducts of benzimidazole with alkyl chloroformates synthesized in this study demonstrated improved stability. As a result, the reactions were successfully carried out using equimolar ratios of the *in situ* generated *N*,*N*-diacyliminium reagents and thymol, leading to the formation of the target products with high yields. Unlike the cited study, the reaction was

not successful when using carvacrol, likely due to steric hindrance caused by the presence of two bulky alkoxycarbonyl substituents.

As with the previously reported benzothiazole-containing compounds [22], the newly synthesized benzimidazole–thymol hybrids (4a–d) were also predicted to exhibit low toxicity (Table 3).

Bhoi *et al.* conducted an *in silico* toxicological analysis of the obtained hybrid compounds containing thymol (Figure 2) using the online tool preADMET, which predicts key toxicological parameters such as mutagenicity, carcinogenicity, and other important properties [11].

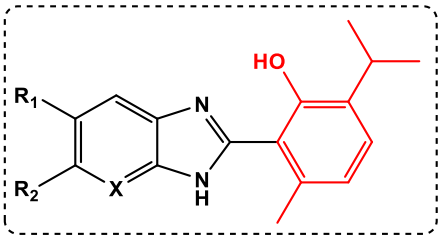


Figure 2. Benzimidazole-thymol hybrids.

The *in silico* modeling-based approach enables a preliminary assessment of the safety and pharmacokinetic properties of novel synthetic compounds. For a more in-depth evaluation of the synthesized benzimidazole derivatives, using the *in silico* machine learning method based on the nearest neighbour approach, implemented in the software system Toxicity Estimation Software Tool (T.E.S.T.), version 5.1.2 [28], the following properties of the compounds presented in Table 3 were successfully predicted: oral rat LD_{50}, mutagenicity and water solubility at 25 °C.

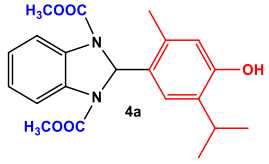
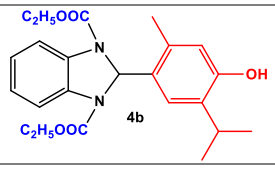
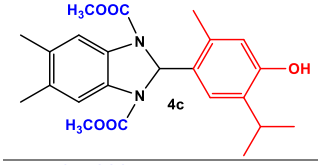
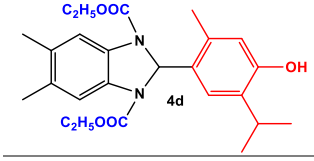
Among the investigated compounds (4a-d), the compound 4a exhibited the highest predicted LD_{50} value (1298.76 mg/kg), suggesting the lowest acute oral toxicity. All compounds were predicted to be non-mutagenic and demonstrated water solubility at 25 °C in the range of 49.19 to 56.37 mg/l.

CONCLUSIONS

In the present study, the optimal reaction conditions for *α*-amidoalkylation of thymol using benzimidazole, 5,6-dimethylbenzimidazole, and acid chlorides, were successfully established. An efficient one-step method was developed for the synthesis of novel hybrid molecules, which was successfully applied using thymol and commercially available thyme essential oil. It was found that the acyliminium reagents retain their stability in the presence of thyme essential oil, allowing the reaction to proceed without formation of undesired side products.

The successfully synthesized novel *N*,*N*-diacylated benzimidazoline–thymol hybrids (4a-d) were evaluated using *in silico* methods. All compounds exhibited the predicted low oral toxicity in rats (high LD_{50} values), negative mutagenicity results, and good water solubility at 25 °C, making them promising candidates for further pharmacological investigations (Table 3).

Table 3. *In silico* toxicity analysis by software system T.E.S.T., version 5.1.2.

Compound (4a-d)	MW, g/mol	Oral rat LD_{50} mg/kg	Mutagenicity	Water solubility at 25 °C mg/l
	384.43	1298.76	Mutagenicity Negative	49.19
	412.49	827.54	Mutagenicity Negative	52.78
	412.49	827.54	Mutagenicity Negative	52.78
	440.54	883.83	Mutagenicity Negative	56.37

Among them, compound 4a showed the highest predicted LD_{50} value (1298.76 mg/kg). Although the present study does not include biological testing, the newly synthesized compounds may serve as a good lead for further pharmacological studies and screening for potential biological activity.

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