Full Paper

Benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidines: Synthesis, Characterization, Antimicrobial Activity, and Incorporation into Solid Lipid Nanoparticles

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Fused triazolothienopyrimidines were prepared from the corresponding 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carbonitrile. These precursors were intern prepared by employing the Gewald's reaction. All the newly synthesized compounds were characterized by spectral and analytical data. Title compounds displayed promising antibacterial and antifungal activities. Compound **3h** which exhibited good antimicrobial activity was incorporated into SLN and characterized for particle size, entrapment efficiency (EE%), scanning electron microscopy (SEM), differential scanning calorimetry (DSC) and *in-vitro* release studies. It showed narrow particle size distribution with high entrapment efficiency. *In-vitro* release study of compound loaded SLNs in phosphate buffer of pH 7.4, exhibited a biphasic pattern with an initial burst and prolonged release over 24 h.

Keywords: Antimicrobial activity / Gewald reaction / *In-vitro* release study / Solid lipid nanoparticles / Triazolothienopyrimidines

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Introduction

Synthesis of novel fused heterocycles is an important task for heterocyclic chemists from various points of view for the development of compounds of pharmacological and industrial importance. Pyrimidine derivatives and heterocyclic annulated pyrimidines continue to attract great interest due to their wide variety of interesting biological activities observed for these compounds, such as antimicrobial [1, 2], anticancer [3], antiviral [4], antitumor [5], and anti-inflammatory activity [6]. Furthermore, many condensed heterocyclic systems, especially when linked to thiophene ring as thienopyrimidine plays an important role in the field of medicinal chemistry [7–9]. The rapid growth in the literature dealing with the synthesis and biological activities of the thienopyrimidine derivatives prompted us to undertake the synthesis of novel fused thienopyridine derivatives.

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Solid lipid nanoparticles (SLNs) possess a combined advantage of overcoming the problems associated with other colloidal carriers. Hence it has attracted much attention in recent years, and is regarded as an alternative carrier system to traditional colloidal systems, such as emulsions, liposomes and polymeric micro particles and nanoparticles [10–12]. Proposed advantages include possibility of controlled drug release and drug targeting, increased drug stability, high drug payload, incorporation of lipophilic and hydrophilic drugs. Furthermore, it doesn't involve biotoxicity of the carrier, and avoids the use of organic solvents and provides the scope of large scale production and sterilization [13]. Many pharmaceutical researchers have prepared SLNs as alternative colloidal therapeutic systems, utilizing different approaches like modified high shear homogenization and ultrasound techniques [10], emulsification–diffusion [14], solvent injection [15], solvent diffusion [16], micro emulsion method [17], and hot homogenization technique [18].

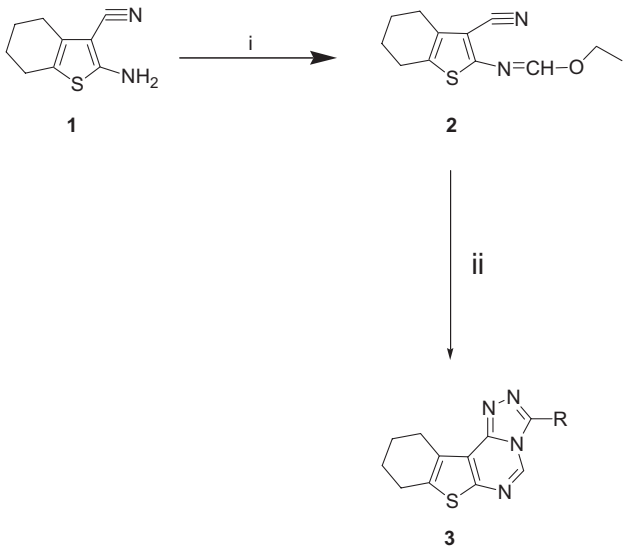
Recently we have reported the synthesis of 1,2,4-triazolo[1,5-*c*]pyrimidine derivatives [2]. Here, we report the synthesis, characterization, and the incorporation into solid lipid nanaoparticles of isomeric 1,2,4-triazolo[4,3-*c*]pyrimidine derivatives.

Result and discussion

Chemistry

The required 2-amino-4,5,6,7-tetrahydro-benzo[*b*]thiophene-3-carbonitrile **1** was prepared by Gewald's reaction as reported in the literature [19, 20]. Formation of 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carbonitrile **1** was characterized by the presence of a band at 2210 cm^{-1} due to a cyano group and ν_{N-H} stretching bands at 3339 and 3190 cm^{-1} in their IR spectra. Furthermore, it was also supported by the presence of D_2O exchangeable broad singlet at δ 7.97 in the ^{1}H-NMR spectrum due to a NH_2 group.

Imidoformate **2** was prepared in excellent yield by treating **1** with triethyl orthoformate at refluxing temperature (Scheme 1). The structure of imidoformate **2** was established by the absence of ν_{N-H} in IR and the presence of a triplet at δ 1.42 and a quartet at δ 4.47 corresponding to protons of the ethoxy group and a peak around δ 8.47 due to N=CH in the ^{1}H-NMR spectrum along with the expected signals. The reaction of imidoformate ester **2** with the appropriate acid hydrazides in refluxing toluene afforded the desired triazole fused thienopyridines (**3**) in moderate yields. The structures of these target compounds were ascertained by their analytical and spectral data. IR spectra of these compounds exhibited bands at 1617 and 1531 cm^{-1} due to C=N and C=C. ^{1}H-NMR spectra displayed the absence of peaks due to ethoxy protons present in compounds **2** and presence of protons due to R substituent in **3**. Finally the structures were confirmed by their mass spectral data.



Reagent and Conditions: i) Triethylorthoformate, reflux, ii) $RCONHNH_2$, AcOH, reflux.R = 9-xanthyl (**3a**), 9-floryl (**3b**), 2-chloro phenyl (**3c**), 3-benzyl (**3d**), 2-naphthyl (**3e**), 4-methyl-thiophen-2-yl (**3f**), pyren-1-yl (**3g**), 4-methyl-furan-2-yl (**3h**).

Scheme 1. Synthesis of 3-aryl-9,10,11,12-tetrahydro[1]benzothieno[3,2-*e*],[1,2,4]triazole[4,3-*c*]pyrimidine (**3**).

Antimicrobial activity

All the newly synthesized compounds were evaluated for their antimicrobial properties [21, 22]. MIC's were recorded as the minimum concentration of compound, which inhibits the growth of tested microorganisms. Compounds exhibited moderate to better activity against all bacteria *viz*., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus subtilis* (Table 1). Remaining compounds **3a**, **3b**, **3c**, **3d**, **3f**, and **3g** exhibited moderate antifungal against *Candida albicans* and *Candida parapsilosis*. Amongst the compound tested, **3h** displayed good antibacterial activity against *S. aureus* and *B. subtilis* but better antifungal activity against *C. albicans* and *C. parapsilosis*. Compound **3e** exhibited good antifungal activity against *C. albicans*.

Table 1. Antibacterial and antifungal activities of the compounds (**3a–h**) as MIC values (μg/mL)

Compounds	*Staphylococcus aureus*	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*	*Candida parapsilosis*
	ATCC 25923	ATCC 6633	ATCC 25922	ATCC 27853	ATCC 10231	ATCC 90018
3a	64	32	32	32	32	32
3b	32	16	32	32	32	32
3c	16	16	32	64	32	32
3d	32	32	32	64	64	32
3e	32	16	32	32	4	16
3f	64	32	32	64	32	32
3g	32	32	32	64	32	32
3h	4	4	16	16	8	8
Ampicillin	0.5	0.5	2	–	–	–
Fluconazole	–	–	–	–	0.5	0.25

R = 9-xanthyl (**3a**), 9-floryl (**3b**), 2-chloro phenyl (**3c**), 3-benzyl (**3d**), 2-naphthyl (**3e**), 4-methyl-thiophen-2-yl, pyren-1-yl (**3g**), 4-methyl-furan-2-yl (**3h**).

Table 2. Compositions of SLN formulations

Formulation code	Concentration of compound (mg)	Concentration of lipid (%)	Concentration of surfactants, 1:1 (%)
3h-SLN-1	10	2.5	2.5
3h-SLN-2	10	5.0	2.5
3h-SLN-3	10	7.5	2.5

SLN formulation

The selected compound **3h** which showed good antimicrobial properties was incorporated into SLN by micro emulsion method. Effect of lipid concentration on particle size was determined.

Particle size and zeta potential

The mean particle size, polydispersity index and zeta potential of colloidal carriers are important characteristics of SLNs from which the stability of drug-loaded SLNs can be predicted. Average particles size, polydispersity, and zeta potential are given in Table 3. All the formulations had shown particle in nanosize range (148.0 ± 1.00 to 176.0 ± 1.00 nm) with narrow size distribution (polydispersity index = 0.105 ± 0.001 to 0.117 ± 0.001). Besides production parameters, lipid matrix, surfactant blend, and viscosity of lipid and aqueous phase influence the outcome of the procedure. Leaving all other parameters constant, in this study the only variable was composition of lipid matrix varying from 2.5% to 7.5%. Effect of lipid concentration on particle size and entrapment efficiency is recorded in Table 3. The results revealed that increasing the lipid content over 2.5–7.5% results in larger particle size [23]. The choice of the emulsifiers and their concentration is of great impact on the quality of the SLN dispersion [24]. Investigating the influence of the emulsifier concentration on the particle size of SLN dispersions, we obtained best results with 2.5% egg lecithin. High concentrations of the emulsifier reduce the surface tension and facilitate the particle partition. The decrease in particle size is connected with a tremendous increase in surface area. The SLNs are stabilized with surfactant mixtures (egg lecithin/Tween 80) to have lower particle sizes and higher storage stability. Use of surfactant mixtures with the aim to have lower particle size are also reported in the literature [23–25]. The measurement of the zeta potential allows predictions about the stability of colloidal aqueous dispersions [26]. Usually, particle aggregation is less likely to occur for charged particles with high zeta potential due to electric repulsion [27]. In general, lipid nanoparticles are negatively charged on the surface [28]. The determination of zeta potential was performed in aqueous SLN stored at room temperature.

Drug entrapment efficiency (EE%)

According to Professor Muller the prerequisite to obtain a sufficient loading capacity was a sufficiently high solubility of the drug in the lipid melt. Relative higher drug EE% was one of the major advantages of SLNs [10]. The EE% of compound loaded SLNs is given in Table 3. The loading capacity of SLN was found to be satisfactorily high. The data showed EE% as high as 86.15 ± 0.60 for some formulation. The entrapment efficiency is lower for the sample with lower lipid concentration (Table 3). It has to be noticed that during the cooling process, the lipid solidifies and the compound is distributed into the shell of the particles, if the concentration of the compound in the melted lipid is well below its saturation solubility [29].

Scanning electron microscopy (SEM)

SEM image of the **3h**-SLN3 derived from Joel JSM 840A has been presented in Fig. 1. SEM confirms that the nanoparticles are irregular in shape. They are smooth, flake like structure and well separated on the surface.

Differential Scanning Calorimetry (DSC)

The thermal curves of GMS bulk lipid and compound showed endothermic peaks at 59.32°C and 194.03°C respectively (Fig. 2). The melting endothermic peaks of the nanoparticles appeared at slightly lower temperature (59.23°C). The decrease in melting temperature of nanoparticles formulated GMS lipid compared with the bulk has been attributed to their small size and presence of surfactants.

Short term Stability study

It has been found that particle size of **3h**-SLN-3 increases by 6 nm and entrapment efficiency was lowered by 3.27%

Table 3. Particle size, polydispersity index, zeta potential and entrapment efficiency

Formulation code	Average diameter (nm)*	Polydispersity index*	Zeta potential (mV)*	Entrapment efficiency (EE%)*
3h-SLN-1	148.0 ± 1.00	0.105 ± 0.001	−22.30 ± 1.10	66.63 ± 1.10
3h-SLN-2	165.0 ± 1.00	0.114 ± 0.001	−24.50 ± 1.10	69.42 ± 0.90
3h-SLN-3	176.0 ± 1.00	0.117 ± 0.001	−26.20 ± 1.00	86.15 ± 0.60

Mean ± SD, $n = 3$

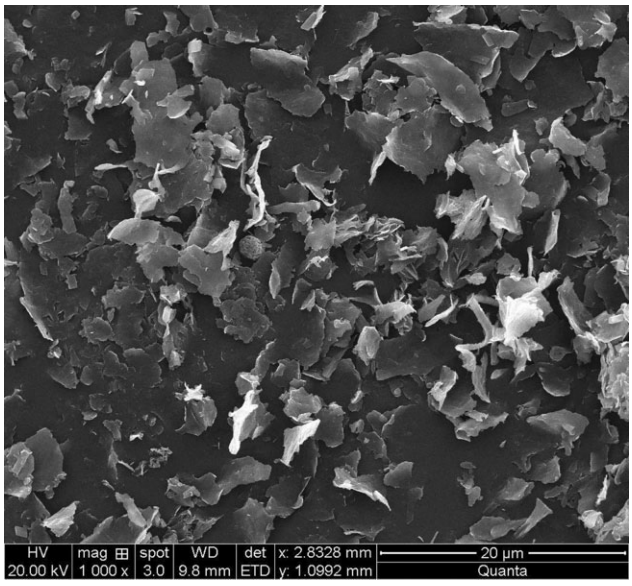


Figure 1. SEM of **3h**-SLN3.

(Table 4) after one month storage at 40°C, 75% RH. Transitions of dispersed lipid from metastable forms to stable form might occur slowly on storage due to small particle size and the presence of emulsifier that may lead to drug expulsion from solid lipid nanoparticles. Therefore lowered entrapment efficiency observed on storage may be due to expulsion during lipid modification [18, 30].

Effect of sterilization

Effect of sterilization on particle size and entrapment efficiency is given Table 5. The high temperature reached during sterilization by autoclaving presumably causes a hot o/w micro emulsion to form in the autoclave, and probably modifies the size of the hot nanodroplets. On subsequent slow cooling, the SLN reformed, but some nanodroplets may coalesce, producing larger SLN than the initial ones. Since SLN are washed before sterilization, amounts of surfactant and cosurfactant present in the hot system are smaller, so that the nanodroplets may be not sufficiently stabilized. In selected Comp-SLN-3 formulation, size of particles increases almost two times after sterilization, but still they are in nanosize. It was found that sterilization by autoclaving has least effect on entrapment efficiency. Therefore, sterilization by autoclaving can be performed for SLNs of GMS stabilized with lecithin and Tween 80 [31].

In-vitro release study

Vertical flow-through Franz diffusion cells and dialysis bag/tubes were used by many research groups for the study of drug release from solid lipid and polymeric nanoparticles and niosomes [32–39]. In order to evaluate the controlled

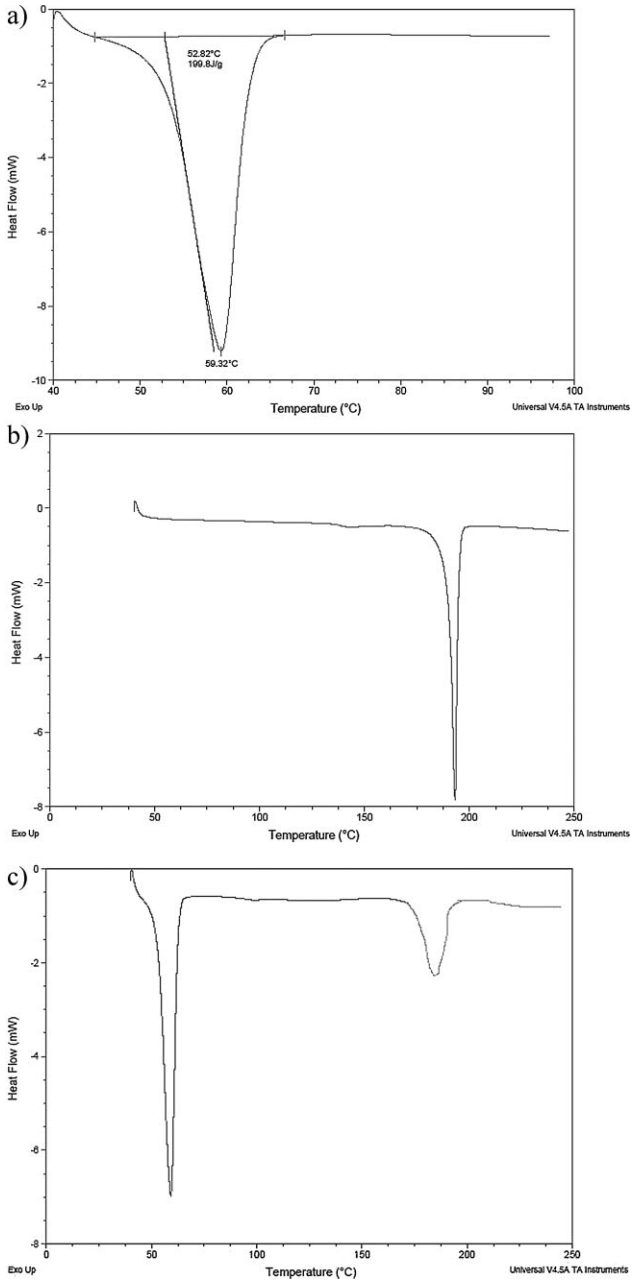


Figure 2. DSC thermograms of (a) bulk lipid (b) compound **3h** (c) compound loaded SLN.

release potential of the investigated formulations, the diffusion of compound from the lipid particles was investigated over 24 h. Each sample was analyzed in triplicate. The results are shown in Fig. 3. The release rate of compound depends on the total concentration of compound in the formulation. Compound is released more quickly when using lower concentration because of the drug-enriched shell model proposed for these particles (**3h**-SLN-1).

Table 4. Effect of time of storage (at 40°C, 75% RH) on particle size and entrapment efficiency

Formulation code	Particle size (nm)*		Entrapment efficiency (%)*	
	Day zero	One month	Day zero	One month
3h-SLN-3	176.0 ± 1.00	182.0 ± 0.76	86.15 ± 0.60	82.88 ± 0.30

*mean ± SD, $n = 3$

Table 5. Effect of sterilization on particle size and entrapment efficiency

Formulation code	Particle size (nm)*		Entrapment efficiency (%)*	
	Before	After	Before	After
3h-SLN-3	176.0 ± 1.00	358.3 ± 0.65	86.15 ± 0.60	83.45 ± 0.60

*mean ± SD, $n = 3$

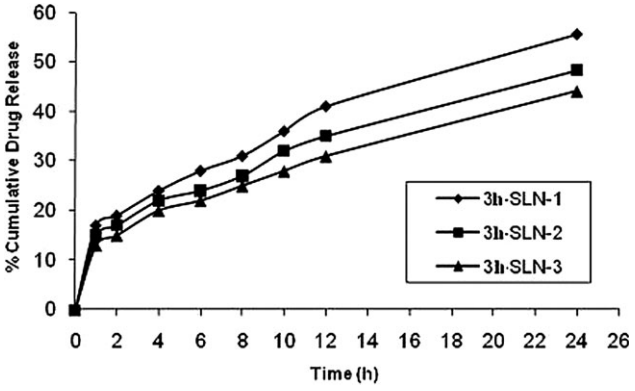


Figure 3. *In-vitro* release study of compound **3h** loaded SLN.

Percentage of compound released from SLNs up to 24 h were in the following order; **3h**-SLN-1 (55.52%), **3h**-SLN-2 (48.23%) and **3h**-SLN-3 (44.12%). The release pattern revealed that there was an initial burst effect followed by a prolonged release of drug. This is because the compound may be located primarily in the shell of the particles. Other factors contributing to a fast release are large surface area, high diffusion coefficient (small molecular size), low matrix viscosity and short diffusion distance of the drug. The compound enriched core is surrounded by a compound free lipid shell. Due to the increased diffusional distance and hindering effects by surrounding solid lipid shell, the compound has a sustained release profile. The formulations were further subjected to release kinetic studies. Release of compound from almost all the SLNs followed Higuchi equation better than the first order equation.

Experimental

General details

Melting points were determined in open capillaries and are uncorrected. The IR spectra were recorded on Nicolet Impact 410 FT IR spectrophotometer (Model-410, USA) using KBr pellets. ^{1}H- and ^{13}C-NMR were recorded on Bruker 300 MHz NMR spectrometer (Model RX-300, Switzerland) in $CDCl_3$ with TMS as internal standard. Mass spectra were recorded on Finnigan MAT (Model MAT8200) spectrometer and elemental analyses were carried out using Heraeus CHN rapid analyzer.

*Synthesis of N-(3-cyano-4,5,6,7-tetrahydro-benzo[b] thiophen-2-yl)-formimidic acid ethyl ester (**2**)*

A solution of **1** (1.33 g, 5 mmol) in triethylorthoformate (12 mL) was heated under reflux for 18 h, excess triethylorthoformate was removed under vacuum. The residue was treated with petroleum ether. The separated solid was filtered and recrystallized with petroleum ether to afford light brown crystals.

Yield 89%, m.p.: 122–124°C, IR (KBr) cm^{-1}: 3090, 2921, 2866, 2212, 1573, 1541. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (t, 3H, CH_3), 1.84 (m, 4H, CH_2), 2.62 (m, 4H, CH_2), 4.44 (q, 2H, CH_2), 7.93 (s, 1H, N=CH); mass *m*/*z*: 234.3 (M^+). Anal. calcd. for $C_{12}H_{14}N_2OS$; C, 61.51; H, 6.02; N, 11.96; S, 13.68. Found: C, 61.02; H, 6.10; N, 11.12; S, 13.34.

*General procedure for the synthesis of 3-aryl-9,10,11,12-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]-pyrimidine (**3a–h**)*

A mixture of **2** (0.26 g, 1 mmol) and the corresponding acid hydrazide ($RCONHNH_2$, 1 mmol) was stirred at room temperature in toluene (10 mL). Then, glacial acetic acid (0.1 mL) was added and the resulting reaction mixture was refluxed for further 12 h. The reaction mixture was washed with water and dried over sodium sulphate. Toluene was removed under reduced pressure to get analytically pure product.

*3-(9H-Xanthen-9-yl)-9,10,11,12-tetrahydro[1] benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (**3a**)*

Yield: 84%, m.p.: 168–170°C. IR (KBr) cm^{-1}: 3047, 2933, 2859, 1612. ^{1}H-NMR ($CDCl_3$) δ: 1.99 (m, 4H, CH_2), 2.93 (t, J = 8 Hz, 2H, CH_2), 3.14 (t, J = 8 Hz, 2H, CH_2), 5.82 (s, 1H, CH), 7.00–7.32 (m, 8H, Ar-H), 9.00 (s, C_5-H pyrimidine proton); mass *m*/*z*: 410.4 (M^+). Anal. calcd. for $C_{24}H_{18}N_4OS$: C, 70.34; H, 4.42; N, 13.65; S, 7.81. Found: C, 70.82; H, 4.90; N, 14.08; S, 7.84.

*3-(9H-Fluoren-9-yl)-9,10,11,12-tetrahydro[1] benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (**3b**)*

Yield: 78%, m.p.: 216–218°C; IR (KBr) cm^{-1}; 3057, 2925, 2853, 1615; ^{1}H-NMR ($CDCl_3$) δ: 1.99 (m, 4H, CH_2), 2.93 (t, J = 8 Hz, 2H, CH_2), 3.24 (t, J = 8 Hz, 2H, CH_2), 5.60 (s, 1H, CH), 7.31–7.85 (m, 8H, Ar-H), 9.02 (s, C_5-H pyrimidine proton); mass *m*/*z*: 394.4 (M^+). Anal. calcd. for $C_{24}H_{18}N_4S$; C, 73.07; H, 4.60; N, 14.20; S, 8.13. Found: C, 73.72; H, 4.20; N, 13.98; S, 8.04.

*3-(2-Chloro-phenyl)-9,10,11,12-tetrahydro[1] benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (**3c**)*

Yield: 88%, m.p.: 161–163°C. IR (KBr) cm^{-1}: 3090, 2921, 2866, 1625, 1573, 1541; ^{1}H-NMR ($CDCl_3$) δ: 1.97 (m, 4H, CH_2), 2.94 (t, $J = 8$ Hz, 2H, CH_2), 3.23 (t, $J = 8$ Hz, 2H, CH_2), 7.40–8.06 (m, 4H, Ar-H), 9.23 (s, C_5-H pyrimidine proton); ^{13}C-NMR δ: 22.4, 23.6, 25.6, 25.8, 115.7, 120.6, 129.5, 129.6, 129.9, 130.3, 135.8, 139.9, 150.1, 153.7, 163.2, 164.4, 166.3; mass *m/z*: 340.8 (M^+). Anal. calcd. for $C_{17}H_{13}ClN_4S$: C, 59.91; H, 3.84; N, 16.44; S, 9.41. Found: C, 59.22; H, 3.56; N, 15.94; S, 9.24.

*3-(3-Benzyl)-9,10,11,12-tetrahydro[1]benzothieno-[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (**3d**)*

Yield: 78%, m.p.: 138–140°C. IR (KBr) cm^{-1}: 3092, 2924, 2868, 1628, 1573, 1541; ^{1}H-NMR ($CDCl_3$) δ: 1.96 (m, 4H, CH_2), 2.94(t, $J = 8$ Hz, 2H, CH_2), 3.24 (t, $J = 8$ Hz, 2H, CH_2), 3.82 (s, 2H, -CH_2-), 7.32–7.45 (m, 5H, Ar-H), 9.19 (s, 1H, C_5-H pyrimidine proton); mass *m/z*: 320.1 (M^+). Anal. calcd. for $C_{18}H_{16}N_4S$: C, 67.47; H, 5.03; N, 17.49; S, 10.01. Found: C, 67.20; H, 5.38; N, 17.98; S,9.90.

*3-(Naphthalen-2-yl)-9,10,11,12-tetrahydro[1] benzothieno[3,2e][1,2,4]triazolo[4,3-c]pyrimidine (**3e**)*

Yield: 78%, m.p.: 227–229°C. IR (KBr) cm^{-1}: 3064, 2927, 2857, 1619; ^{1}H-NMR (300 MHz,$CDCl_3$) δ: 1.98 (m, 4H, CH_2), 2.93 (t, $J = 8$ Hz, 2H, CH_2), 3.25 (t, $J = 8$ Hz, 2H, CH_2), 7.56–8.44 (m, 6H, Ar-H), 9.21 (d, $J = 9$ Hz, 1H, C_2 naphthalene), 9.27 (s, C_5-H, pyrimidine proton); mass *m/z*: 356.4 (M^+). Anal. calcd. for $C_{21}H_{16}N_4S$; C, 70.76; H, 4.52; N, 15.72; S, 9.00. Found: C, 70.12; H, 4.20; N, 15.92; S, 9.04.

*3-(3-Methyl-thiophen-2-yl)-9,10,11,12-tetrahydro[1] benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (**3f**)*

Yield: 80%, m.p.: 202–204°C. IR (KBr) cm^{-1}: 3054, 2932, 2855, 1617. ^{1}H-NMR ($CDCl_3$) δ: 1.98 (m, 4H, CH_2), 2.36 (s, 3H, CH_3), 2.93 (t, $J = 8$ Hz, 2H, CH_2), 3.25 (t, $J = 8$ Hz, 2H, CH_2), 7.18 (d, 1H, $J = 6$ Hz, thiophenic proton), 7.95 (d, 1H, $J = 6$ Hz, thiophenic proton), 9.11 (s, C_5-H pyrimidine proton); mass *m/z*: 326.3 (M^+). Anal. calcd. for $C_{16}H_{14}N_4S_2$: C, 58.87; H, 4.32; N, 17.16; S, 19.65. Found: C, 58.28; H, 4.10; N, 17.32; S, 19.82.

*3-(Pyren-1-yl)-9,10,11,12-tetrahydro[1]benzothieno-[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (**3g**)*

Yield: 63%, m.p.: 216–218°C. IR (KBr) cm^{-1}: 3065, 2928, 2857, 1619; ^{1}H-NMR ($CDCl_3$) δ: 1.98 (m, 4H, CH_2), 2.93 (t, $J = 8$ Hz, 2H, CH_2), 3.25 (t, $J = 8$ Hz, 2H, CH_2), 7.71–8.04 (m, 9H, Ar-H), 9.13 (s, C_5-H pyrimidine proton); mass *m/z*: 434.5 (M^+). Anal. calcd. for $C_{27}H_{22}N_4S$: C, 74.63; H, 5.10; N, 12.89; S, 7.38. Found: C, 74.42; H, 5.56; N, 12.08; S, 7.64.

*3-(3-Methyl-furan-2-yl)-9,10,11,12-tetrahydro[1] benzothieno[3,2e][1,2,4]triazolo[4,3-c]pyrimidine (**3h**)*

Yield: 88%, m.p.: 196–198°C. IR (KBr) cm^{-1}: 3065, 2928, 2856, 1620; ^{1}H-NMR ($CDCl_3$) δ: 1.98 (m, 4H, CH_2), 2.21 (s, 3H, CH_3), 2.93 (t, $J = 8$ Hz, 2H, CH_2), 3.24 (t, $J = 8$ Hz, 2H, CH_2), 7.08 (d, $J = 6$ Hz, 1H), 7.45 (d, $J = 6$ Hz, 1H), 9.14 (s, C_5-H, pyrimidine proton); mass *m/z*: 310.3 (M^+). Anal. calcd. for $C_{16}H_{14}N_4OS$: C, 61.92; H, 4.55; N, 18.05; S, 10.33. Found: C, 61.62; H, 4.85; N, 17.95; S, 10.03.

SLN formulation

Preparation of triazolothienopyrimidines loaded SLNs

SLNs formulations were prepared from a warm o/w microemulsion technique [23]. The synthesized compound **3h** was dispersed in molten lipid (70°C), warm aqueous solution of egg lecithin was added to melted lipid-drug mixture (at 70°C) in presence of the co-surfactant Tween 80 under stirring. The warm microemulsion was then added carefully drop wise into ice cold water (2–3°C) with continuous stirring (T25 basic Ultra Turrax, IKA, USA). The ratio between the microemulsion and the dispersion medium was about 1:10. The dispersion was subjected to ultrasonication for a period of 10 min to form nanosuspension (Table 2).

Particle size analysis and zeta potential

Size and zeta potential of molecule loaded SLN were measured by Photon Correlation Spectroscopy (PCS) using zetasizer 3000 HSA (Malvern, U.K.). Samples were diluted appropriately with the aqueous phase of the formulation for the measurements and the pH of diluted samples ranged from 6.8 to 7.0.

Entrapment efficiency

The entrapment efficiency of the synthesized molecule was determined by measuring the concentration of free drug in the dispersion medium [23]. The sample was centrifuged at 4000 rpm for 30 min. The amount of free drug was determined in the clear supernatant by UV spectrophotometer using supernatant of non-loaded nanoparticles as basic correction. The amount of incorporated compound was determined by subtracting amount of initial compound from the free compound. The entrapment efficiency was calculated by the following equation [24]:

$$EE(\%) = \left(\frac{W_{\text{initial compound}} - W_{\text{free compound}}}{W_{\text{initial compound}}}\right) \times 100$$

where $W_{\text{initial compound}}$ is the weight of the initial compound and $W_{\text{free compound}}$ the weight of the free compound.

Scanning electron microscopy (SEM)

The morphological examination of **3h**-SLN3 was performed by scanning electron microscopy (Joel JSM 840A, Japan). Cleaned brass specimen studs were used for mounting the samples. Wet solvent paint was applied on these studs and while the paint was wet, the pellets were placed on each stud and allowed to dry. The sample was observed by SEM.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis of the drug, bulk lipid and nanoparticles was conducted using a differential scanning calorimeter (DSC Q20 V24.4 Build 116, TA Instruments, USA) set at a heating rate of 10°C/min.

Short-term stability study

The selected SLN formulation was stored at 40°C, 75% RH, in a stability chamber (Thermo Lab., Mumbai) for a period of one month and average particle size and entrapment efficiency was determined.

Effect of sterilization

To observe the effect of sterilization on particle size and entrapment efficiency, selected SLN formulation was autoclaved at 121°C for 20 min.

In-vitro release study:

In-vitro release studies were performed using modified Franz diffusion cells [19] having a surface area of 2.545 cm^2 and 75 mL of capacity. Dialysis membranes (LA 401) with a pore size of 2.4 nm and a molecular weight cutoff of 12 000–14 000 (HIMEDIA) were used. The membrane was soaked in distilled water for 12 h before mounting in cell. SLN equivalent to 5 mg of compound was placed in the donor compartment and the receptor compartment was filled with dialysis medium (phosphate buffer of pH 7.4, 75 mL). The content of the cell was stirred with the help of magnetic stirrer at 37°C. At fixed time intervals, 1 mL of the sample was withdrawn from the receiver compartment through the side tube. Fresh phosphate buffer of pH 7.4 was placed to maintain constant volume. Samples were analyzed by UV spectrophotometry.

Antimicrobial activity

Minimum inhibitory concentration (MIC) values for the synthesized compounds were determined by using the broth micro dilution method [21, 22]. Two Gram-positive (*S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633) and two Gram-negative (*E. coli* ATCC25922 and *P. aeruginosa* ATCC 27853) bacteria were used as quality control strains. For determining anti yeast activities of the compounds, the following reference strains were used: *Candida albicans* ATCC10231 and *Candida parapsilosis* ATCC 90018. Ampicillin trihydrate and fluconazole were used as standard antibacterial and antifungal agents, respectively. Fluconazole was dissolved in sterile distilled water, ampicillin trihydrate in phosphate buffer (pH 8) and the stock solutions of the synthesized compounds were prepared by dissolving in dimethyl sulfoxide (DMSO) and distilled water (50%) at a concentration of 2048 μg/mL. Twofold dilutions of the synthesized compounds were prepared (1024, 512...2 μg/mL), and twofold dilutions of the reference compounds were prepared at 64–0.125 μg/mL. All bacteria were cultivated in Mueller–Hinton agar (Merck). The bacteria inoculums were prepared in Mueller–Hinton broth (Merck) which had been kept at 36°C overnight and were diluted with broth to give a final concentration of 5×10^5 cfu/mL. All fungi were cultivated in Sabouraud dextrose agar (Merck). The fungi inoculums were prepared in Sabouraud liquid medium (oxoid) which had been kept at 36°C overnight and were diluted with RPMI-1640 medium with L-glutamine buffered with 3-[*N*-morpholino]-propane sulfonic acid (MOPS) at pH 7 to give a final concentration of 2.5×10^3 cfu/mL. The microplates were incubated at 36°C and read visually after 24 h, except for *Candida* species after 48 h. The incubation chamber was kept humid. At the end of the incubation period, MIC values were recorded as the lowest concentrations of the substances that gave no visible turbidity. The DMSO diluents at a maximum final concentration of 12.5% had no effect on the microorganism's growth. The (MIC) were noted (Table 1).

Conclusion

The present study reports the synthesis of novel fused tetracyclic thienotriazolopyrimidines from the corresponding precursor 2-amino-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carbonitrile. Compound **3h** which exhibited good antimicrobial activity was incorporated in SLN and showed narrow particle size distribution with high entrapment efficiency. Furthermore, it revealed that optimization of SLN formulation is an important factor in achieving increased duration of action and also release from the dosage form to achieve sustained effect.

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