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REGULAR ARTICLE

Synthesis and biological evaluation of 3,6-dialkylsubstituted-[1,2,4] triazolo[3,4-b][1,3,4]thiadiazoles

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Abstract. A series of 3,6-dialkyl-[1,2,4] triazolo[3,4-b][1,3,4]thiadiazole (10) analogues were prepared through multistep synthesis and evaluated them for their antimicrobial and cytotoxic activities. Synthesis of target compounds was carried out using undecenoic acid as starting material, which is the renewable product of castor oil. The key step in the synthesis was formation of triazolo [3,4-b][1,3,4]thiadiazole using various free fatty acids in presence of $POCl_3$. It was observed that the undecenyl based triazolothiadiazole with butyl (**6a**), hexyl (**6b**) and lauryl (**6f**) derivatives exhibited promising antimicrobial activity against the tested strains. Particularly, Compound **6a** exhibited the most promising activity with MIC value 3.9 μg/mL against most of the tested strains. It also showed potent minimum bactericidal concentration activity with MIC value 7.8 μg/mL against the tested strains. Cytotoxicity data revealed that most of the tested compounds revealed cytotoxic activity, Compounds **6b, 6d, 6f, 6g, 6h** and **6i** against SKOV3, **6d, 6e, 6f, 6g, 6h, 6i** and **6j** against MCF-7 and **6c, 6d, 6e,6g, 6h, 6i** and**6j** against B16–F10 cell lines exhibited significant activities with IC_{50} values ranged between 13.67 and 18.62 μM. Interestingly, all the compounds were non toxic against Chinese hamster ovary cell (CHO-K1) normal cell.

Keywords. Di alkyltriazolothiadiazole; cytotoxicity; antimicrobial; butyl; hexyl; lauryl.

1. Introduction

The prevalence of microbial infections has increased considerably in recent years. A bacterial and fungal infection remains a serious threat to human life because of its emerging resistance to existing antibiotic drugs, which is an increasing public health problem. Consequently, there is an urge to discover novel antimicrobial agents with potent activity against drug resistant microorganisms. In addition, cancer is a major public health problem that can affect almost every tissue in the human body and poses a great challenge to medical science. Thus, intense efforts are needed to discover new compounds with improved selectivity and activity by chemical modifications. The development of new antimicrobial and anticancer therapeutic agents is one of the fundamental goals in medicinal chemistry. Therefore, continuous efforts are needed to develop novel compounds with anticancer and antimicrobial activities.

Fatty acids are ubiquitous molecules typically found bound to glycerol, sugars and phosphate and other head groups to form lipids. Fatty acids are the important components of all of these lipids. Fatty acids and their derivatives are known for their biological activities such as antimicrobial,[1,2] antifungal,[3] and pesticidal[4] ones. These fatty acid analogs have been found to be

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associated with diverse biological activities such as anti-inflammatory,[5] antioxidant,[6] antifeedant,[7] antiparasitic,[8] antimicrobial[9] and neuroprotective.[10] Literature studies reported that a variety of modified fatty acids are significant molecules in the treatment of cancers.[11–13] Undecenoic acid derivatives were found to exhibit promising biological activities such as antifungal, antibacterial, antiviral and anticancer ones.[11,14]

In addition, 1,2,4-triazoles are a group of heterocyclic compounds exhibiting a wide variety of biological activities such as antibacterial, antifungal,[15] antitubercular,[16] anticancer,[17] anticonvulsant,[18] antiinflammatory,[19] analgesic[20] and molluscicidal properties.[21–24] 1,2,4-triazoles are good starting materials for the synthesis of some interesting N-bridged heterocycles due to their ambident nucleophilic centers. Triazolothiadiazoles and triazolothiadiazines are a class of fused heterocyclic compounds, which attracted great interest in medicinal chemistry owing to their wide range of pharmacological activities such as antifungal,[25,26] antibacterial,[27,28] antiviral,[29] antihelmentic,[30,31] antitumour,[32] analgesic[33] and anti-inflammatory ones.[34,35]

Based on the above facts, in the present study a series of 3,6-dialkylsubstituted-[1,2,4] triazolo[3,4-b][1,3,4]thiadiazoles were synthesized using various fatty acids and evaluated for biological activities.

2. Experimental

2.1 *Materials and general synthetic procedures*

All the chemicals used in these schemes were of analytical grade and they were obtained from different commercial sources and used without any further purification. Reactions were monitored on micro TLC plates (coated with TLC grade silica gel, obtained from Merck). Column chromatography was performed by using silica gel (100–200 mesh) procured from Qualigens (India) using freshly distilled solvents. All the ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Bruker Avance (for ^{1}H-NMR at 300 MHz, 400 MHz, 500 MHz and for ^{13}C-NMR at 75 MHz, 100 MHz, 125 MHz) spectrometer, using TMS $\delta = 0$ ppm and δ 77.00 ppm as internal standard for chemical shifts (δ) in $CDCl_3$ at 25 °C. The chemical shift values are presented in ppm (parts per million) units. Mass spectra were recorded with HRMS. IR spectra were recorded in chloroform on a Perkin-Elmer FT-IR spectrum BX.

2.1a *Synthesis of methyl undec-10-enoate (2)*: To a stirred solution of undec-10-enoic acid (73.45 mmol) in methanol (100 mL), a few drops of concentrated H_2SO_4 were added. The reaction mixture was refluxed for 10 h. Progress of the reaction was monitored by micro TLC. After completion the reaction, methanol was removed under reduced pressure and water was added and the title compound was extracted with ethyl acetate, dried over anhydrous sodium sulphate and concentrated under vacuum to afford the title compound.

^{1}H NMR (300 MHz, $CDCl_3$): (δ, ppm) = 5.75−5.85 (m, -CH = CH_2-, 1H), 4.91–5.01 (m, -CH = CH_2-, 2H), 3.66(s, -OCH_3, 2H), 2.28–2.31(t, -CH_2-, J = 7.4 Hz, 2H), 2.01–2.06(m, -CH_2-, 2H), 1.59–1.65(m, -CH_2-, 2H), 1.26–1.39(m, -$(CH_2)_5$-, 10H).
ESI-mass: $[M + H]^+ m/z = 199$.

2.1b *Synthesis of undec-10-enehydrazide (3)*: To a stirred solution of methyl undec-10-enoate (2) (59.93 mmol) in ethanol (90 mL), hydrazine hydrate (269.68 mmol) was added. The reaction mixture was refluxed for about 10 h. Progress of the reaction was monitored by micro TLC. After completion of the reaction, the solvent was evaporated under reduced pressure, ice water (50 mL) was added and the mixture was stirred for 15 min. The solid obtained was filtered and dried under vacuum to yield undec-10-enehydrazide as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ(ppm) = 5.75−5.85 (m, -CH = CH_2-, 1H), 4.91–5.01 (m, -CH = CH_2, 2H), 2.67–3.01(broad-s, -NH_2, 2H), 2.12–2.16(t, -CH_2-, J = 7.3 Hz, 2H), 2.00–2.06 (m, -CH_2-, 2H), 1.59–1.66(m, -CH_2-, 2H), 1.25–1.38(m, -$(CH_2)_5$-, 10H).
ESI-mass: $[M + H]^+ m/z = 199$.

2.1c *Synthesis of potassium 2-(undec-10-enoyl) hydrazine-1-carbodithioate (4)*: Potassium hydroxide pellets (106.54 mmol) were dissolved in ethanol (40 mL). To this solution, undec-10-enehydrazide (53.27 mmol), carbon disulfide (117.19 mmol) were added successively and the contents were stirred at room temperature for 8 h. Progress of the reaction was monitored by micro TLC. After completion of the reaction, diethyl ether (100 mL) was added to the reaction mixture and stirred for 10 min. After filtration, potassium 2-(undec-10-enoyl) hydrazine-1-carbodithioate was obtained as an off-white solid.

2.1d *Synthesis of 4-amino-5-(dec-9-en-1-yl)-4H-1, 2, 4-triazole-3-thiol (5)*: Hydrazine hydrate (45.38 mmol) was added to potassium 2-(undec-10-enoyl) hydrazine-1-carbodithioate (45.38 mmol) and the contents were refluxed for 5 h. Progress of the reaction was monitored by micro TLC. After completion of the reaction, the reaction mixture was acidified with concentrated hydrochloric acid. The obtained precipitate was filtered and dried under vacuum to obtain the crude compound which was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 85: 15, v/v) as an off white solid.

ESI-mass: $[M + H]^+ m/z = 255$.

2.1e *General procedure for the synthesis of bridged compounds* **(6a-j)**: A mixture of 4-amino-5-(dec-9-en-1-yl)-4H-1, 2, 4-triazole-3-thiol (0.1 mol), various fatty acids (0.1 mol) and phosphorus oxychloride (10 L) was refluxed for

6 h. Progress of the reaction was monitored by micro TLC. After completion of the reaction, reaction mixture was cooled to room temperature and poured onto crushed ice and the title compound was extracted with ethyl acetate, dried over anhydrous sodium sulphate. The crude compound was subjected to silica gel column chromatography to get the required product.

2.1f *3-(Dec-9-en-1-yl)-6-propyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole* **(6a)**: The crude compound was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 20: 80, v/v) as an off-white semi solid with 67% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ(ppm) = 5.75−5.85(m, 1H, CH_2-*CH* = *C*H_2), 4.91–5.01(m, 2H, CH_2-*CH* = *C*H_2), 3.02–3.06(t, J = 7.58 Hz, 2H, CH_2), 2.92–2.96(t, J = 7.45 Hz, 2H, CH_2), 2.00–2.06(m, 2H, CH_2), 1.81–1.90(m, 2H, CH_2), 1.63–1.66(m, 2H, CH_2), 1.25–1.41(m, 10H, $(CH_2)_5$), 1.05-1.09(t, J = 7.33Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 169.3, 153.3, 148.0, 139.0, 114.0, 34.1, 33.6, 29.0, 28.9, 28.8, 26.6, 24.9, 21.8, 13.3; IR ($CHCl_3$ ν_{max} cm^{-1}): 3422, 2925, 2854, 1468, 1216, 758; HR-MS (ESI) m/z [M+H$^+$]: calc for $C_{16}H_{26}N_4S$ is 306.1878, found 307.19478 ($C_{16}H_{27}N_4S$).

2.1g *3-(Dec-9-en-1-yl)-6-pentyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole* **(6b)**: The crude compound was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 20: 80, v/v) as an off white semi solid with 63% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ(ppm) =5.76–5.84(m, 1H, CH_2-*CH* = CH_2), 4.91–5.01(m, 2H, CH_2-*CH* = *C*H_2), 3.02–3.05(t, J = 7.62Hz, 2H, CH_2), 2.95–2.97(t, J = 7.62Hz, 2H, CH_2), 1.99–2.05(m, 2H, CH_2), 1.78-1.89(m, 4H, CH_2), 1.25–1.43(m, 14H, $(CH_2)_7$), 0.91–0.94(t, J = 7.01 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 168.9, 152.9, 147.7, 138.7, 113.7, 33.7, 33.3, 28.7, 28.6, 28.4, 26.3, 24.6, 21.5, 13.0; IR ($CHCl_3$ ν_{max} cm^{-1}): 3417, 2925, 2854, 1630, 1468, 1217, 769; HR-MS (ESI) m/z [M+H$^+$]: calc for $C_{18}H_{30}N_4S$ is 334.2191, found 335.22604 ($C_{18}H_{31}N_4S$).

2.1h *3-(Dec-9-en-1-yl)-6-heptyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole* **(6c)**: The crude compound was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 20: 80, v/v) as an off-white semi solid with 66% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ(ppm) = 5.75−5.85(m, 1H, CH_2-*CH* = CH_2), 4.91–5.01(m, 2H, CH_2-*CH* = *C*H_2), 3.02–3.06(t, J = 7.70Hz, 2H, CH_2), 2.93–2.97(t, J = 7.58Hz, 2H, CH_2), 1.98–2.06(m, 2H, CH_2), 1.77–1.90(m, 4H, CH_2), 1.25–1.44(m, 18H, $(CH_2)_9$), 0.87–0.91(t, J = 6.84Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 169.6, 153.6, 148.4, 139.3, 114.3, 34.4, 34.0, 29.3, 29.2, 29.1, 26.9, 25.2, 22.2, 13.6; IR ($CHCl_3$ ν_{max} cm^{-1}): 3418, 2924, 2853, 1958, 1711, 1629, 1468, 1217, 771; HR-MS (ESI) m/z [M+H$^+$]: calc for $C_{20}H_{34}N_4S$ is 362.2504, found 363.25755 ($C_{20}H_{35}N_4S$).

2.1i *3-(Dec-9-en-1-yl)-6-nonyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole* **(6d)**: The crude compound was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 20: 80, v/v) as an off-white semi solid with 70% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ(ppm) = 5.76−5.84(m, 1H, CH_2-*CH* = CH_2), 4.91–5.01(m, 2H, CH_2-*CH* = *C*H_2), 3.02–3.05(t, J = 7.78Hz, 2H, CH_2), 2.94–2.97(t, J = 7.62Hz, 2H, CH_2), 1.99–2.05(m, 2H, CH_2), 1.78–1.89(m, 4H, CH_2), 1.25–1.43(m, 22H, $(CH_2)_{11}$), 0.91–0.94(t, J = 7.01Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 169.9, 153.3, 148.1, 139.0, 114.1, 34.0, 33.7, 31.8, 29.6, 29.3, 29.0, 28.8, 28.4, 26.6, 22.6, 14.0; IR ($CHCl_3$ ν_{max} cm^{-1}): 3418, 2924, 2853, 1719, 1628, 1467, 1377, 1217, 1091, 771; HR-MS (ESI) m/z [M+H$^+$]: calc for $C_{22}H_{38}N_4S$ is 390.2817, found 391.28885 ($C_{22}H_{39}N_4S$).

2.1j *3,6-Di(dec-9-en-1-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole* **(6e)**: The crude compound was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 20: 80, v/v) as an off white semi solid with 65% yield. ^{1}H NMR (400 MHz, $CDCl_3$): 5.75–5.85(m, 2H, CH_2-*CH* = CH_2), 4.91–5.01(m, 4H, CH_2-*CH* = *C*H_2), 3.02–3.06(t, J = 7.70 Hz, 4H, CH_2), 2.93–2.97(t, J = 7.58 Hz, 4H, CH_2), 1.95–2.04(m, 4H, CH_2), 1.76–1.88(m, 4H, CH_2), 1.25–1.42(m, 16H, $(CH_2)_8$); ^{13}C NMR (125 MHz, $CDCl_3$): δ(ppm) = 169.5, 153.3, 148.0, 139.0, 138.9, 114.1, 114.0, 33.6, 32.4, 32.2, 29.3, 29.0, 28.9, 28.7, 28.4, 26.6, 24.9, 17.8; IR ($CHCl_3$ ν_{max} cm^{-1}): 3423, 3016, 2926, 2854, 1473, 1215, 757, 667; HR-MS (ESI) m/z [M+H$^+$]: calc for $C_{23}H_{38}N_4S$ is 402.2817, found 403.28827 ($C_{23}H_{39}N_4S$).

2.1k *3-(Dec-9-en-1-yl)-6-undecyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole* **(6f)**: The crude compound was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 20: 80, v/v) as an off white semi solid with 71% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ(ppm) = 5.76−5.84(m, 1H, CH_2-*CH* = CH_2), 4.91–5.00(m, 2H, CH_2-*CH* = *C*H_2), 3.02–3.05(t, J = 7.62Hz, 2H, CH_2), 2.94–2.97(t, J = 7.62Hz, 2H, CH_2), 2.01–2.05(m, 2H, CH_2), 1.77–1.89(m, 4H, CH_2), 1.26–1.44(m, 26H, $(-CH_2-)_{13}$) 0.86–0.89(t, J = 6.71Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 169.6, 153.0, 147.8, 138.7, 113.7, 33.7, 33.4, 31.5, 29.3, 29.0, 28.7, 28.4, 28.1, 26.3, 22.3, 13.7; IR ($CHCl_3$ ν_{max} cm^{-1}): 3419, 3015, 2925, 2854, 1472, 1215, 759, 667; HR-MS (ESI) m/z [M+H$^+$]: calc for $C_{24}H_{42}N_4S$ is 418.3130, found 419.31976 ($C_{24}H_{43}N_4S$).

2.1l *3-Decyl-6-tridecyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole*(**6g**): The crude compound was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 20: 80, v/v) as an off white solid with 69% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ(ppm) = 5.76−5.84(m, 1H, CH_2-*CH* = CH_2), 4.91–5.01(m, 2H, CH_2-*CH* = *C*H_2), 3.23–3.26 (t, J = 7.62 Hz,

2H, CH_2), 3.05–3.08 (t, J = 7.62Hz, 2H, CH_2), 2.01–2.05(m, 2H, CH_2), 1.82–1.95 (m, 4H, CH_2), 1.25–1.44 (m, 30H, (-CH_2-)$_{15}$) 0.86–0.89(t, J = 6.71 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 170.5, 153.3, 148.1, 139.0, 114.1, 33.7, 32.3, 31.8, 33.9, 29.6, 29.3, 29.0, 28.8, 28.4, 26.6, 24.8, 22.6, 14.0; IR ($CHCl_3$ ν_{max} cm^{-1}): 3652, 3423, 2925, 2854, 1958, 1735, 1639, 1518, 1470, 1215, 994, 769, 667; HR-MS (ESI) m/z [M+H$^+$]: calc for $C_{26}H_{46}N_4S$ is 446.36724, found 447.36692 ($C_{26}H_{47}N_4S$).

2.1m *3-(Dec-9-en-1-yl)-6-pentadecyl-[1,2,4]triazolo [3,4-b][1,3,4]thiadiazole* **(6h)**: The crude compound was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 20: 80, v/v) as an off white solid with 76% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ(ppm) = 5.76−5.84(m, 1H, CH_2-*CH* = CH_2), 4.91–5.01(m, 2H, CH_2-*CH* = *CH_2*), 3.02–3.05(t, J = 7.62 Hz, 2H, CH_2), 2.94–2.97(t, J = 7.62 Hz, 2H, CH_2), 2.01-2.05(m, 2H, CH_2), 1.77–1.89(m, 4H, CH_2), 1.25-1.45(m, 34H, (-CH_2-)$_{17}$) 0.86-0.89(t, J = 6.71 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 169.9, 153.3, 148.1, 139.0, 114.1, 34.0, 33.7, 31.8, 29.6, 29.3, 29.0, 28.8, 28.4, 26.6, 22.6, 14.0; IR ($CHCl_3$ ν_{max} cm^{-1}): 3418, 2925, 2854, 1640, 1518, 1471, 1215, 769, 667; HR-MS (ESI) m/z [M+H$^+$]: calc for $C_{28}H_{51}N_4S$ is 475.38289, found 475.38266 ($C_{28}H_{51}N_4S$).

2.1n *3-(Dec-9-en-1-yl)-6-heptadecyl-[1,2,4]triazolo [3,4-b][1,3,4]thiadiazole* **(6i)**: The crude compound was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 20: 80, v/v) as an off white solid with 72% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ(ppm) = 5.75−5.85(m, 1H, CH_2-*CH* = CH_2), 4.91–5.01(m, 2H, CH_2-*CH* = *CH_2*), 3.02–3.06(t, J = 7.58 Hz, 2H, CH_2), 2.93–2.97(t, J = 7.58 Hz, 2H, CH_2), 2.00–2.05(m, 2H, CH_2), 1.76–1.90(m, 4H, CH_2), 1.25–1.45(m, 38H, (-CH_2-)$_{19}$) 0.86–0.89(t, J = 6.60 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 169.6, 153.3, 148.1, 139.0, 114.1, 34.0, 33.7, 32.3, 31.8, 29.6, 29.3, 29.0, 28.8, 28.4, 26.6, 24.9, 22.6, 14.0; IR ($CHCl_3$ ν_{max} cm^{-1}): 3418, 2925, 2854, 1958, 1732, 1640, 1470, 1215, 993, 769, 666; HR-MS (ESI) m/z [M+Na$^+$]: calc for $C_{28}H_{56}N_4SNa$ is 503.41179, found 503.41393($C_{28}H_{56}N_4SNa$).

2.1o *3-(Dec-9-en-1-yl)-6-(heptadec-8-en-1-yl)-[1,2,4]triazolo[3,4b][1,3,4]thiadiazole* **(6j)**: The crude compound was subjected to silica gel column chromatography and the required product was eluted in a solvent mixture (Hexane: EtOAc, 20: 80, v/v) as an off-white semi solid with 78% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ(ppm) = 5.76−5.84 (m, 1H, CH_2-*CH* = CH_2), 5.34–5.35 (m, 2H, -*CH* = *CH*-), 4.91–5.01(m, 2H, CH_2-*CH* = *CH_2*), 3.02–3.05(t, J = 7.58 Hz, 2H, CH_2), 2.93–2.96(t, J = 7.58 Hz, 4H, CH_2), 1.94–2.05(m, 4H, CH_2), 1.77–1.89 (m, 4H, CH_2), 1.25–1.42(m, 30H, (-CH_2-)$_{15}$) 0.86–0.89(t, J = 6.60Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 169.6, 153.3, 148.1, 139.1, 130.1, 129.9, 114.1, 33.7, 32.3, 31.8, 29.6, 29.2, 29.0, 28.7, 28.4, 26.7, 25.0, 22.6, 14.0; IR ($CHCl_3$ ν_{max} cm^{-1}): 3423, 2925, 2854, 1958, 1734, 1639, 1518, 1471, 1215, 993, 757, 666; HR-MS (ESI) m/z [M+H$^+$]: calc for $C_{30}H_{53}N_4S$ is 501.39854, found 501.39838 ($C_{30}H_{53}N_4S$).

2.2 *General procedure for biological evaluation*

2.2a *Antibacterial and antifungal assays*: The antibacterial and antifungal activities of the synthesized compounds were determined using well diffusion method[36] against different pathogenic bacterial strains such as *Micrococcus luteus* MTCC 2470, *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS-16 MTCC 2940, *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453 and *Klebsiella planticola* MTCC 530 along with different *Candida* strains such as *Candida albicans* MTCC 183, *C. albicans* MTCC 227, *C. albicans* MTCC 854, *C. albicans* MTCC 1637, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, *C. albicans* MTCC 3958, *C. albicans* MTCC 4748, *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744, *C. aaseri* MTCC 1962, *C. glabrata* MTCC 3019, *C. krusei* MTCC 3020 and *Issatchenika hanoiensis* MTCC 4755. All these bacterial and fungal strains were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. The pathogenic reference strains were seeded on the surface of the media Petri plates, containing Muller-Hinton agar with 0.1 mL of previously prepared microbial suspensions individually containing 1.5×10^8 cfu mL^{-1} (equal to 0.5 McFarland standard). Wells of 6.0 mm diameter were prepared in the media plates using a cork borer and the synthesized compounds dissolved in 10% DMSO at a dose range of 125–0.97 μg were added in each well under sterile conditions in a laminar air flow chamber. Standard antibiotic solutions of ciprofloxacin (bacterial strains) and miconazole (*Candida* strains) at a dose range of 125–0.97 μg well^{-1}, served as positive controls, while the well containing DMSO served as negative control. The plates were incubated for 24 h at 30 °C and the well containing the least concentration showing the inhibition zone is considered as the minimum inhibitory concentration. All experiments were carried out in duplicates and mean values are represented.

2.2b *Minimum bactericidal and fungicidal concentration (MBC) assays*: Bactericidal and fungicidal assay (NCCLS, 2000) was performed in sterile 2.0 mL microfuge tubes against a panel of pathogenic bacterial strains, including *Micrococcus luteus* MTCC 2470, *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS-16 MTCC 2940, *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453, *Klebsiella planticola* MTCC 530 and for fungicidal activity different *Candida* strains were cultured overnight in Mueller Hinton broth. Serial dilutions of test compounds were prepared in Mueller Hinton broth with different concentrations ranging from 0 to 150 μg mL^{-1}. To the test compounds, 100 μL of overnight

cultured bacterial and *Candida* suspensions were added to reach a final concentration of 1.5×10^8 cfu mL^{-1} (equal to 0.5 McFarland) and incubated at 37 °C for 24 h. After 24 h of incubation, the minimum bactericidal or fungicidal concentration (MBC/MFC) was determined by sampling 10 μL of suspension from the tubes onto Mueller Hinton agar plates and were incubated for 24 h at 37 °C to observe the growth of test organisms. MBC/MFC are the lowest concentration of test compound required to kill a particular bacterium/*Candida* strain. All the experiments were carried out in duplicates.

2.2c *Biofilm inhibition assay*: The test compounds were screened in sterile 96 well polystyrene microtiter plates using the modified biofilm inhibition assay,[37] against a panel of pathogenic bacterial strains including *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS-16 MTCC 2940 and *Klebsiella planticola* MTCC 530, which were cultured overnight in tryptone soy broth (supplemented with 0.5% glucose). The test compounds of predetermined concentrations ranging from 0 to 250 μg/mL were mixed with the bacterial suspensions having an initial inoculum concentration of 5×10^5 CFU/mL. Aliquots of 100 μL were distributed in each well and then incubated at 37 °C for 24 h under static conditions. The medium was then discarded and washed with phosphate buffered saline to remove the non-adherent bacteria. Each well of the microtiter plate was stained with 100 μL of 0.1% crystal violet solution followed by 30 min incubation at room temperature. Later the crystal violet solution from the plates was discarded, thoroughly washed with distilled water for 3 to 4 times and air dried at room temperature. The crystal violet stained biofilm was solubilised in 95% ethanol (100 μL) and the absorbance was recorded at 540 nm using TRIAD multimode reader (Dynex Technologies, Inc, Chantilly, VA, USA). Blank wells were employed as background check. The inhibition data were interpreted from the dose-response curves, where IC_{50} value is defined as the concentration of inhibitor required to inhibit 50% of biofilm formation under the above assay conditions. All the experiments were carried out in triplicates and the values are indicated as mean ± S.D.

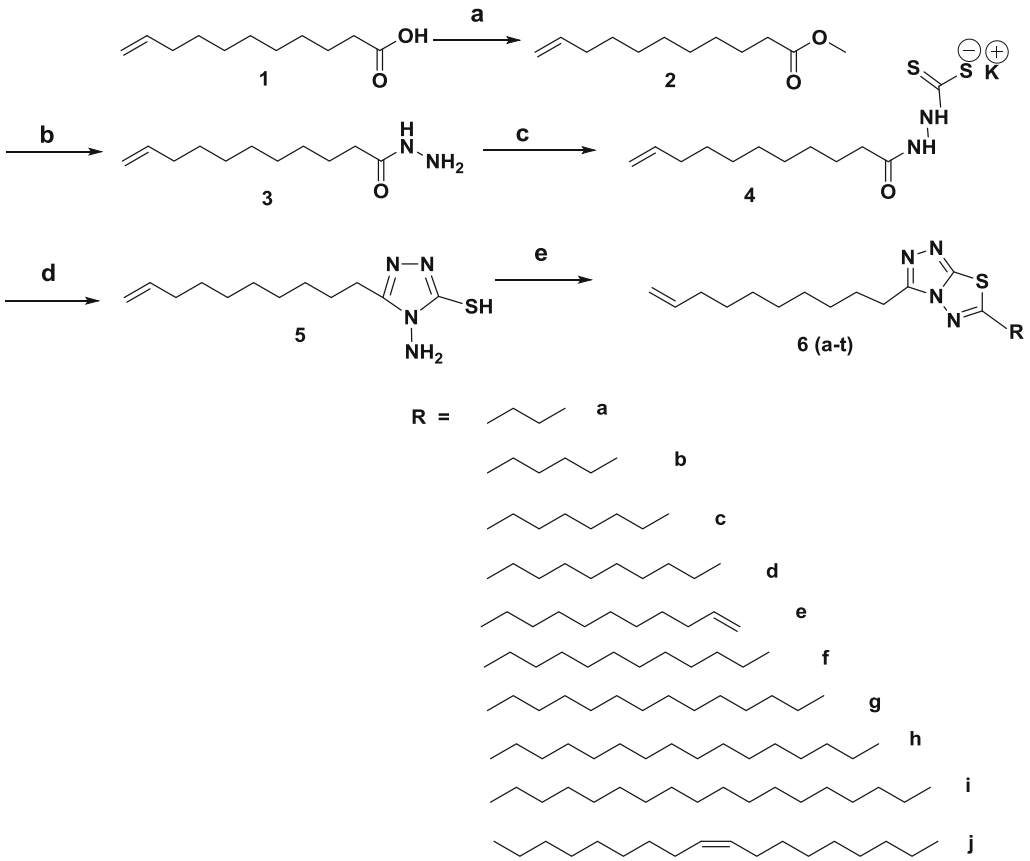
2.2d *Cytotoxicity assay*: Cytotoxicity assay (MTT) was evaluated for the synthesized compounds as per the method reported in the earlier study.[38] Four different cancer cell lines and one normal cell line namely, HeLa Homo sapiens cervix adenocarcinoma (ATCC® CCL-2.1™), B16-F10 Mouse skin melanoma (ATCC® CRL¬6475™), SKOV3 Human Ovarian cancer (ATCC® HTB¬77™), MCF7 Human Breast Adenocarcinoma ((ATCC® HTB-22™) and CHO-K1-Chinese hamster ovary cells, Normal Cell line (ATCC® CCL-61™) were obtained from the ATCC (Bethesda, MD, USA) and maintained in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 incubator. After seeding of cells in 96 well culture plate, allowed to attach properly. Test compounds of different concentrations ranging from 1 to 50 μM were added in triplicates and incubated for 24 h. The cells were then incubated with MTT (0.5 mg/mL) for 3 h and to dissolve the insoluble formazan crystals 100 μL DMSO was added to each well. Finally, the absorbance of the plates was measured using a Synergy H1 multi-mode plate reader, USA. Doxorubicin was used as a positive control for the comparison.

3. Results and Discussion

The target compounds were synthesized as outlined in Scheme 1. Undec-10-enoic acid was converted to the methyl ester by using methanol and few drops of concentrated sulphuric acid. Undec-10-enoic acid methyl ester was treated with hydrazine hydrate to get Undec-10-enehydrazide, which was further reacted with carbon disulfide in ethanolic potassium hydroxide to yield corresponding dithiocarbazinate in good yield. Dithiocarbazinate was directly reacted with hydrazine hydrate under refluxing conditions to yield triazole. Condensation of triazole with various fatty acids in presence of $POCl_3$ yielded triazolothiadiazoles (6a-t). The synthesized compounds were characterized by ^1H, ^{13}C NMR, ESI-MS, HRMS and IR spectral analysis.

All the synthesized compounds **6a–j** were directly screened for their *in vitro* antimicrobial activity against different gram-positive strains such as *Staphylococcus aureus* MTCC 96, *Bacillus subtilis* MTCC 121, *S. aureus* MLS16 MTCC 2940, *Micrococcus luteus* MTCC 2470, and gram-negative bacterial strains such as *Klebsiella planticola* MTCC 530, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453, as well as fungi such as *Candida albicans* MTCC 3017.

Compound **6a** (against *Staphylococcus aureus* MTCC 96, *Bacillus subtilis* MTCC 121, *S. aureus* MLS16 MTCC 2940, *Micrococcus luteus* MTCC 2470, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453 strains) exhibited the most promising activity with MIC value 3.9 μg/mL. Compounds, **6b** (against *Staphylococcus aureus* MTCC 96 and *Pseudomonas aeruginosa* MTCC 2453 strains) and **6f** (against *Staphylococcus aureus* MTCC 96, *Bacillus subtilis* MTCC 121 and *Micrococcus luteus* MTCC 2470 strains) exhibited good activity with MIC value 7.8 μg/mL. Compound **6a** showed antifungal activity against *Candida albicans* MTCC 3017 with the MIC value 3.9 μg/mL. It is noteworthy that, the antimicrobial activities in this study depend on the alkyl chain lengths of triazolothiadiazole at C-6 position. Interestingly, our results corroborate with the previous findings where heterocyclic compounds having alkyl chain moiety showed significant biological activities.[39] Compound **6a** with a basic 1,2,4-triazolo[3,4-b]1,3,4-thiadiazole scaffold having butyl



Scheme 1. Reagents and conditions: (a) MeOH, H_2SO_4, reflux, 10 h; (b) Hydrazine hydrate, EtOH, reflux, 10 h; (c) CS_2, KOH, EtOH, RT, 8 h; (d) Hydrazine hydrate, reflux, 5 h; (e) R-COOH, $POCl_3$, reflux, 6 h.

moiety at C-6 position and undecyl moiety at C-3 position showed the prominent activity. Compounds having hexyl (**6b**) and lauryl (**6f**) at C-6 position and undecyl moiety at C-3 position displayed good activities.

Compound **6a** (butyl) showed potent minimum bactericidal concentration activity with MIC value 7.8 μg/mL against the tested strains. Compounds **6b** (hexyl) against *Staphylococcus aureus* MTCC 96 (MIC value 7.8 μg/mL) and *Pseudomonas aeruginosa* MTCC 2453 (MIC value 7.8 μg/mL) and **6f** (lauryl) against *Staphylococcus aureus* MTCC96, *Bacillus subtilis* MTCC 121 and *Micrococcus luteus* MTCC 2470 (MIC value 7.8 μg/mL) displayed promising activities.

Further, all these compounds were screened for antibiofilm activity against various strains, which are important nosocomial pathogens encountered in medical establishments and devices and have the ability to form biofilms. The results to this regard are summarized in Table 3, which clearly reveal that few of the compounds exhibited moderate activities towards all the tested strains.

The cytotoxicity of all the synthesized compounds were screened against four human cancer cell lines namely, HeLa Homo sapiens cervix adenocarcinoma (ATCC® CCL-2.1™), B16-F10 Mouse skin melanoma (ATCC® CRL¬6475™), SKOV3 Human Ovarian cancer (ATCC® HTB¬77™), MCF7 Human Breast Adenocarcinoma ((ATCC® HTB-22™) and CHO-K1-Chinese hamster ovary cells, Normal Cell line (ATCC® CCL-61™) using MTT assay. Doxorubicin was used as a positive control. IC_{50} values of the test compounds for 24 h on each cell line was calculated and presented in Table 4.

Majority of the synthesized compounds exhibited cytotoxicity on all the tested cell lines (Table 4). Compounds **6b, 6d, 6f, 6g, 6h** and **6i** showed significant activities against SKOV3 with the IC_{50} values ranged between 13.80 to 18.62 μM. Compounds **6d, 6e, 6f, 6g, 6h, 6i** and **6j** displayed significant activities against MCF-7 cell line with IC_{50} values ranged between 13.67 to 18.02 μM and compounds **6c, 6d, 6e, 6g, 6h, 6i** and **6j** exhibited significant activities against B16-F10 cell line with IC_{50} values ranged from 13.80 to 18.49 μM. Remaining compounds showed moderate activity against HeLa cell line (IC_{50} values, 22.56 to 57.50 μM). All the compounds were non toxic against Chinese hamster ovary cell (CHO-K1) normal cell.

4. Conclusions

In conclusion, a series of di-alkyl 1, 2, 4-triazolo [3, 4-b] [1, 3, 4] thiadiazoles were synthesized and their antimicrobial activity and cytotoxicity were investigated. It was observed that, butyl, hexyl and lauryl derivatives exhibited good antimicrobial activities. Butyl analogue showed potent minimum bactericidal concentration activity. Hexyl, decyl, undecenyl, lauryl, myristyl, palmityl, stearyl and oleyl-based derivatives exhibited significant activities against SKOV3 and MCF-7 cell lines.

Supplementary Information

Tables 1-3 and Figures 1-20 is available at www.ias.ac.in/chemsci.

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