Original Article

JHSMR Journal of Health Science and Medical Research

Antimicrobial and Antibiofilm Activities of Synthetic Lawsone Derivatives Containing *N*-Substituted 1,2,3-Triazole Against Dental Caries Pathogens

Pichayaporn Ratti, D.D.S.[1], Jutharat Manuschai, D.D.S., M.Sc.[1], Jiraporn Kara, B.Sc., M.Sc.[2,3], Supawadee Naorungroj, D.D.S., M.Sc., Ph.D.[1], Luelak Lomlim, B.Pharm., M.Pharm., Ph.D.[2,3]

[1]Department of Conservative Dentistry, Faculty of Dentistry, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.

[2]Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.

[3]Phytomedicine and Pharmaceutical Biotechnology Excellent Center (PPBEC), Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.

Received 25 July 2022 ● Revised 28 September 2022 ● Accepted 28 September 2022 ● Published online 9 November 2022

Abstract:

Objective: Presently, one of the most common oral diseases is dental caries, which is a biofilm-mediated disease. Lawsone methyl ether (LME) has shown promising antibacterial activity due to its 1,4-napthoquinone structure. Recently, a 1,2,3-triazole scaffold has been used in the structural modification of potential antimicrobial agents. To develop novel anticaries agents, the structure modification of 1,4-napthoquinone with *N*-substituted 1,2,3-triazole, therefore, may be a candidate.

Material and Methods: LME was used as a lead compound, and three new lawsone derivatives were prepared by two-step reactions. Their antimicrobial effects against three dental caries pathogens; including *S. mutans*, *L. casei*, and *A. naeslundii* were investigated, using the microdilution technique (0.78-100 μg/mL). A growth curve assay was performed to assess the effects of compounds on the growth kinetics of bacteria. Moreover, the effect of synthetic lawsone derivatives on the biofilm formation of *S. mutans* was also evaluated by crystal violet assay.

Results: Overall, *S. mutans* was most sensitive to lawsone derivatives (minimum inhibitory concentration (MIC)=1.56-50 μg/mL), followed by *A. naeslundii* and *L. casei*: corresponding to their growth curves. Lawsone derivatives, at the

This paper was from the 1st Annual Health Research International Conference (AHR-iCON, August 1-2, 2022).
Contact: Luelak Lomlim, B.Pharm., M.Pharm., Ph.D.
Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90100, Thailand.
E-mail: luelak.l@psu.ac.th

J Health Sci Med Res 2023;41(2):e2022906
doi: 10.31584/jhsmr.2022906
www.jhsmr.org

concentration of 1/2 MIC and 1/4 MIC, inhibited 12-hour *S. mutans* biofilm formation by 86.0-98.0%. The inhibitory effect decreased with decreasing concentrations and increasing incubation times.

Conclusion: Synthetic lawsone derivatives have an inhibitory effect on the growth of three tested cariogenic bacteria, and the biofilm formation of *S. mutans*. The compounds exhibited anti-cariogenic bacterial strains and satisfying anti-biofilm formation effects on *S. mutans.*

Keywords: antibacterial activity, anticaries, dental biofilm, lawsone derivatives, naphthoquinone, triazole

Introduction

Dental caries is a biofilm-mediated disease that causes localized demineralization and damage to dental hard tissue. Untreated dental caries in permanent teeth is the most common health overburdening condition worldwide.[1] Primary etiological factors that play a critical role in developing dental caries are microorganisms in dental biofilm; especially facultatively and obligately anaerobic bacteria.[2-3] *Streptococcus mutans* is the primary etiological pathogen of dental caries, which plays an important role in developing cariogenic biofilms.[4] *Actinomyces naeslundii* is another early colonizer that has been implicated in the formation of root caries lesions.[5] *Lacticaseibacillus casei* is an acidogenic and aciduric bacteria, which is also well-known as an etiological pathogen of dental caries found in caries lesions.[6]

Lawsone (2-hydroxy-1,4-naphthoquinone) is a bioactive, natural substance found in *Lawsonia inermis* that has exhibited antibacterial and antifungal properties.[7-9] The fundamental pharmacophore responsible for its antimicrobial activity is the *p*-quinone moiety. The mitochondrial respiratory chain of the bacteria is the target of lawsone's antimicrobial and antifungal actions. The *p*-quinone moiety undergoes a redox reaction with oxygen to produce reactive oxygen species (ROS), which can cause intracellular damage and apoptosis. During the Michael addition reaction with bacterial biomolecules, the quinone ring also acts as an electrophile, disrupting their normal function.[9-10]

The incorporation of a five-membered 1,2,3-triazole moiety to the 1,4-naphthoquinone scaffold has resulted in potent antimicrobial and antifungal analogues.[11-12] In addition, many naturally occurring compounds derived with triazole moiety showed potent antimicrobial activity; for example, flustramine C-inspired pyrroloindoline-3-triazole amides[13], indole-triazole amide conjugates[14], oroidin-triazole conjugates[15], 2-aminoimidazole triazole conjugates[16], TAGE-triazole conjugates[17], pyrazolo-[3,4-b]pyridine-triazole conjugates[18], triazole containing naamine A and isonaamine A mimics[19], and triazole derivatives of geraniol and farnesol[20] were found to inhibit the biofilm formation of several Gram-negative and Gram-positive bacteria.

Inspired by the incorporation of 1,2,3-triazole functionality in the 1,4-naphthoquinone and other natural scaffolds, in this ongoing project for the discovery of new anticaries agents, 3 lawsone derivatives containing *N*-substituted 1,2,3-triazole were designed and synthesized. These were then evaluated for their anticariogenic pathogens and antibiofilm activity. To our knowledge, none of the naphthoquinone-containing 1,2,3-triazole derivatives have been investigated for anticaries properties. The design of lawsone derivatives containing *N*-substituted 1,2,3-triazole may provide opportunities for the development of novel anticaries agents.

Material and Methods

Chemicals used in the preparation of the compounds were purchased from Sigma-Aldrich or Merck AG. The progress of reactions and the purities of the compounds were detected by thin layer chromatography (TLC), on silica gel 60 F254 aluminum sheets (Merck AG). Melting points were recorded using the Mel-TEMP II laboratory devices, USA. Infrared spectroscopy (IR) spectroscopy were performed on a Perkin Elmer spectrum and absorption bands were shown in cm^{-1}. ^{1}H-Nuclear Magnetic Resonance (NMR) and ^{13}C-NMR spectra were recorded by a BRUKER/AVANCETM NEO using deuterated chloroform ($CDCl_3$) or deuterated dimethyl sulfoxide (d_6-DMSO) as solvent. NMR spectra splitting patterns were designated as follows: s: singlet; d: doublet; m: multiplet. High-resolution Mass spectrometry (HR-MS) spectra were recorded on a Thermo Finnigan MAT 95XL.

Synthesis of lawsone derivatives containing *N*-substituted 1,2,3-triazole

Synthesis of lawsone derivatives containing *N*-substituted 1,2,3-triazole started from the synthesis of 2-(prop-2-ynyloxy)naphthalene-1,4-dione (compound **1**)[21], and synthesis of azide derivatives (**3a-3c**), and subsequently the synthesis of the 1,2,3-triazole analogues (compound **4a-4c**), via copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction.[22] (Scheme 1)

Synthesis of 2-(prop-2-ynyloxy)naphthalene-1,4-dione (1)

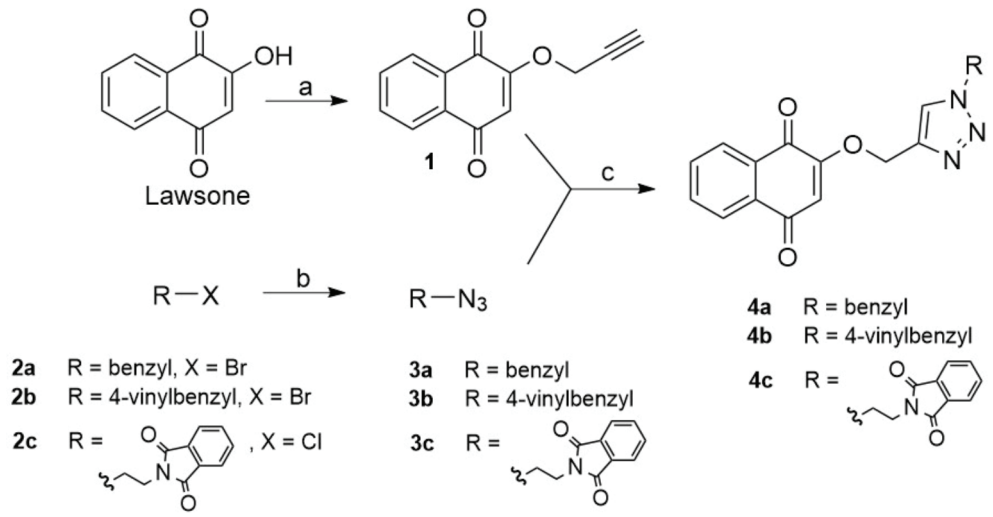
A solution of lawsone (100 mg, 0.58 mmol) in *N,N*-dimethyl formamide was stirred at room temperature under a nitrogen atmosphere. K_2CO_3 (79.5 mg, 0.58 mmol) was added to the solution and the reaction mixture was stirred for 15 minutes. Propargyl bromide (61.5 mg, 0.58 mmol) was added to the reaction mixture and the mixture was further stirred for 72 hours. At completion of the reaction, the product was extracted by ethyl acetate (3x30 mL). The combined organic phase was washed with water (3x30 mL) and dried over anhydrous Na_2SO_4. The solid was filtered off, and the filtrate was concentrated under reduced pressure to produce the crude product. The product was then purified by column chromatography and recrystallization from hexane/methanol.

General method for the synthesis of azides (3a-3c)

Sodium azide (3.16 mmol) was added into a solution of the corresponding alkyl halide (R-X) (1.5 mmol) in DMSO (10 mL), and the reaction mixture was stirred at room temperature under a nitrogen atmosphere for 24 hours. Iced water was poured into the reaction mixture, and the product was partitioned in diethyl ether (3x30 mL). The combined organic phase was washed with 5.0% sodium bicarbonate (1x30 mL) and distilled water (2x30 mL). The excess water was removed from the organic phase by addition of anhydrous Na_2SO_4. The solvent was removed by mean of rotary evaporation. The product was purified by silica gel column chromatography using dichloromethane:hexane (60:40) as eluent.

General method for the synthesis of lawsone derivatives containing *N*-substituted 1,2,3-triazole (4a-4c)

2-(Prop-2-ynyloxy)naphthalene-1,4-dione (0.34 mmol) and the corresponding azide (0.34 mmol) were dissolved in ethanol (25 mL), and the solution was stirred at room temperature. Then, $CuSO_4$ (0.1 M, 66.37 µL) and copper powder (0.08 mmol) was added into the reaction mixture. Stirring was continued for 24 hours. The solid was filtered off and the filtrate was concentrated with a rotary evaporator. The residue was further purified by silica gel column chromatography using a mixture of dichloromethane/



Scheme 1 Synthesis pathway of the 1,2,3-triazoles; (a) K_2CO_3/ propargyl bromide/ DMF/ r.t. 72 h; (b) NaN_3/ DMSO/ r.t. 24 h (c) $CuSO_4$/ Copper powder/ EtOH/ r.t. 24 h

hexane (for **4a**), dichloromethane/methanol (for **4b**), or ethyl acetate/hexane (for **4c**) as the mobile phase. The product was recrystallized from methanol/hexane as a yellow solid.

Predictive physicochemical parameters

The SwissADME program (http://www.swissadme.ch) was used to determine Mw, consensus log P, number of hydrogen bond donors and acceptors, rotatable bonds, topological polar surface area (tPSA), water solubility and skin permeation of the lawsone derivatives.[23-24]

Bacterial strains and growth conditions

For all the experiments, bacterial strains used in this study included: *Streptococcus mutans* (DMST 41283), *Lacticaseibacillus casei* (TISTR 1463), and *Actinomyces naeslundii* (TISTR 2426). All strains were grown on an agar media for two days before being transferred to liquid media under the same growth conditions used throughout the study. *S. mutans* and *A. naeslundii* were grown on BHI agar plates (HiMedia Laboratories, Mumbai, India) at 37°C in the presence of 5.0% CO_2; additionally, *L. casei* was grown on MRS agar plates (HiMedia Laboratories, Mumbai, India) at 37°C in the presence of 5.0% CO_2.

Overnight cultures of each strain were subcultured and grown until they reached the mid-exponential phase, so as to prepare bacterial cultures for all experiments. To verify the number of viable cells, colony-forming unit (CFU) counts of bacterial suspension were performed for each experiment using the drop plate method. The estimated numbers at the mid-exponential phase of each strain of viable cells were: 10^8 CFU/mL for *S. mutans* (6 hours) and *L. casei* (12 hours) and 10^7 CFU/mL for *A. naeslundii* (19 hours).

Antimicrobial activity

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values, using the microtiter broth dilution method and growth curve assay in 96-well microtiter plates, were used to determine

antimicrobial activities of lawsone derivatives containing *N*–substituted 1,2,3–triazole. Stock solutions of the synthetic compounds were prepared in 5 mL vials by being dissolved in DMSO 20.0% and ethanol 4.0% to a final concentration of 400 µg/mL. Then, the serial dilutions for synthetic compounds **4a–4c** were made; ranging from 0.195–100 µg/mL Chlorhexidine (CHX) was used as a positive control for antibacterial activity as it is commonly used as an antiseptic mouthwash, which possesses broad –spectrum antimicrobial activity.[25] Broth solutions were used as a negative control.

Bacterial cultures were diluted to approximately 10^5 CFU/mL utilizing a sterile broth. From this suspension, 100 µL was inoculated in each well, and incubated at 37°C for 24 hours. The lowest concentrations of agents that completely inhibited bacterial growth in the wells (the first clear well) were taken as the MIC. Bacterial culture media from wells with no increased turbidity were collected and placed on agar plates for 24 hours of incubation. The MBCs were identified by the lowest concentration, which resulted in negative bacterial growth. The experiment was independently repeated three times to confirm activity.

The growth curve assay was used to determine the effects of synthetic compounds **4a–4c** on growth kinetics. Similar to the microtiter broth dilution technique, bacterial strains (10^5 CFU/mL) were placed in 96–well microtiter plates and incubated using different concentrations of synthetic compounds **4a–4c**: as described above. A microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, US) was used to record the absorbance value of each well, at a wavelength of 620[26], 600[27], and 660[28] nm for *S.mutans*, *L. casei,* and *A. naeslundii,* respectively, every 2 hours until the bacterial growth reached the stationary phase of each strain. Then, graphs as a function of time in hours on the X–axis versus optical density on the Y–axis, were plotted to obtain the growth curves of bacteria.

Antibiofilm activity

The bacterial suspension, 10^5 CFU/mL of *S. mutans* 50 µL, was placed in 96–well microtiter plates and incubated in 1/2–1/8 MIC; with the presence of 5.0% (w/v) sucrose. Wells without synthetic compounds **4a–4c** served as controls. The media and unbound cells were decanted after 12 and 24 hours of incubation. The remaining planktonic cells were gently rinsed away with distilled water. The attached cells (biofilms) were stained with 0.1% crystal violet for 15 minutes at room temperature. After two rinses with distilled water, 95.0% ethanol was added and the plates were shaken for 10 minutes to allow for the full release of the dye. At wavelength 595 nm, the absorbance of extracted crystal violet in ethanol was measured. The inhibition percentages were calculated using the following formula:

$$\%\ \text{inhibition} = 100 - \left(\frac{\text{sample OD}}{\text{control OD}} \times 100\right)$$

Statistical analysis

Line graphs were used to display the changes in absorbance value of bacteria with the presence of synthetic compounds **4a–4c** over time (bacterial growth curves). The Kruskal–Wallis H test was used to determine the difference in the percentage of 12–hour and 24–hour biofilm inhibition among sub–MIC concentrations of each synthetic compound. The software STATA version 13.1 (StataCorp, College Station, Texas) was used for the analysis of the data.

Results

Synthesis of lawsone derivatives (4a–4c)

The target triazole derivatives can be synthesized via 3–step synthesis. In every step, the intermediates and final products were prepared in high yields, and their chemical structures were confirmed by IR, ^{1}H–NMR, ^{13}C–NMR spectroscopy and HR–MS.

2-(prop-2-ynyloxy)naphthalene-1,4-dione (1) Yellow solid. (73.1%); m.p. 149-151°C; IR (cm^{-1}, neat): 3252.1, 3054.1, 2923.8, 1681.9, 1605.7, 1458.4, 1257.4, 1016.2, 722.2, 696.3; ^{1}H-NMR (ppm, $CDCl_3$): 2.65 (1H, *s*), 4.81 (2H, *s*), 6.36 (1H, *s*), 7.74 (2H, *m*), 8.07 (1H, *d*), 8.14 (1H, *d*); ^{13}C-NMR (ppm, $CDCl_3$): 56.74, 75.44, 78.20, 111.61, 126.24, 126.75, 131.05, 131.89, 133.48, 134.38, 158.05, 179.83, 184.71; HR-MS (m/z, $[M+1]^+$): 213.0531 (calcd for $C_{13}H_8O_3$, 212.0473)

Benzyl azide (3a) colorless liquid (89.6%); IR (cm^{-1}, neat): 3338.5, 3032.7, 2931.1, 2097.6, 1455.3, 1256.2, 737.6, 699.1; ^{1}H-NMR (ppm, $CDCl_3$): 4.33 (2H, s, 1-CH2), 7.30-7.39 (5H, m, aromatic H); ^{13}C-NMR (ppm, $CDCl_3$): 54.82, 128.23, 128.32, 128.85, 135.37; HR-MS (m/z, $[M+1]^+$): 134.0951 (calcd for $C_7H_7N_3$, 133.0643)

1-(azidomethyl)-4-vinylbenzene (3b) brown solid (93.0%); m.p. 284-285°C; IR (cm^{-1}, neat): 3341.5, 3088.7, 2978.7, 2099.0, 1609.2, 1447.0, 1273.1, 1046.8, 822.5, 767.8; ^{1}H-NMR (ppm, DMSO-d_6): 4.42 (2H, s), 5.26, 5.29 (1H, d, J=11.8 Hz), 5.82-5.89 (1H, d, J=18.6 Hz), 6.71-6.77 (1H, m), 7.33, 7.35 (2H, d, J=8.1 Hz), 7.48, 7.50 (2H, d, J=8.2 Hz); ^{13}C-NMR (ppm, DMSO-d_6): 53.70, 115.16, 126.88, 129.17, 135.60, 136.59, 137.44; HR-MS (m/z, $[M+1]^+$): 160.0372 (calcd for $C_9H_9N_3$, 159.0796)

2-(2-azidoethyl)isoindoline-1,3-dione (3c) white solid (98.2%); m.p. 82-83°C; IR (cm^{-1}, neat): 3011.7, 2919.2, 2332.1, 1681.6, 1650.0, 1607.9, 1438.3, 1258.8, 1201.2, 1012.9, 778.0,723.9; ^{1}H-NMR (ppm, $CDCl_3$): 3.58-3.61 (2H, t, J=6.0 Hz), 3.89-3.91 (2H, t, J=.06 Hz), 7.73-7.75 (2H, dd, J=3.0, 5.5 Hz), 7. 68, 7.88 (2H, dd, J=3.0, 5.5 Hz); ^{13}C-NMR (ppm, $CDCl_3$): 36.84 48.95, 123.45, 131.82, 134.17, 168.00, 137.44; HR-MS (m/z, $[M+1]^+$): 217.0720 (calcd for $C_{10}H_8N_4O_2$, 216.0647)

2-((1-benzyl-1*H*-1,2,3-triazol-4-yl)methoxy) naphthoquinone-1,4-dione (4a) Yellow solid. (97.2%); m.p. 160-161°C; IR (cm^{-1}, neat): 3067.5, 2929.3, 16842, 1607.9, 1456.5, 1242.6, 1009.9, 782.0, 723.7; ^{1}H-NMR (ppm, DMSO-d_6): 5.23 (2H, s), 5.63 (2H, s), 6.60 (1H, s,), 7.31-7.39 (5H, m), 7.79-7.87 (2H, m), 7.96-7.99 (2H, m), 8.36 (1-H, s); ^{13}C-NMR (ppm, DMSO-d_6): 53.07, 62.51, 111.05, 125.64, 125.68, 126.23, 128.13, 128.34, 128.92, 130.96, 131.61, 133.78, 134.64, 136.01, 141.25, 159.08, 179.59, 184.63; HR-MS (m/z, $[M+1]^+$): 346.1186 (calcd for $C_{20}H_{15}N_3O_3$, 345.1113)

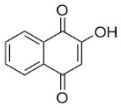
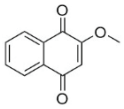
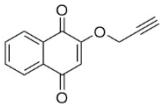
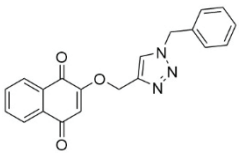
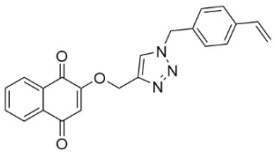
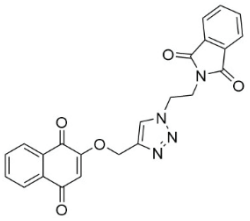
2-((1-(4-vinylbenzyl)-1*H*-1,2,3-triazol-4-yl) methoxy)naphthoquinone-1,4-dione (4b) Yellow solid. (69.6%); m.p. 128-129°C; IR (cm^{-1}, neat): 3136.7, 3006.4, 1686.4, 1605.0, 1471.9, 1240.9, 1010.3, 774.1, 717.5; ^{1}H-NMR (ppm, DMSO-d_6): 5.25-5.29 (3H, m), 5.64 (2H, s), 5.82, 586 (1H, d, *J*=17.6 Hz), 6.62 (1H, s), 6.70-6.76 (1H, m), 7.32, 7.33 (2H, d, *J*=8.0 Hz), 7.48, 7.49 (2H, d, *J*=8.0 Hz), 7.81-7.88 (2H, m), 7.97-8.00 (2H, m), 8.38 (1H, s); ^{13}C-NMR (ppm, DMSO-d_6): 53.12, 62.81, 111.36, 115.40, 125.99, 126.03, 126.58, 126.99, 128.83, 131.28, 131.93, 134.14, 135.00, 135.85, 136.51, 137.56, 141. 85, 159.41, 179.94, 185.01; HR-MS (m/z, $[M+1]^+$): 372.1343 (calcd for $C_{22}H_{17}N_3O_3$, 371.1270)

2-(2-(4-((1,4-dioxo-1,4-dihydronaphtalen-2-yloxy)methyl)-1*H*-1,2,3-triazol-1-yl)ethyl) isoindoline-1,3-dione (4c) Yellow solid. (61.4%); m.p. 244-245°C; IR (cm^{-1}, neat): 3140.4, 2927.8, 1713.0, 1607.5, 1395.5, 1241.3, 782.8, 720.5; ^{1}H-NMR (ppm, DMSO-d_6): 3.99-4.01 (2H, t, *J*=5.7 Hz), 4.66-4.68 (2H, t, *J*=5.7 Hz), 5.22 (2H, s), 6.55 (1H, s), 7.75-7.87 (6H, m), 7.95-8.00 (2H, m), 8.35 (1H, m); ^{13}C-NMR (ppm, DMSO-d_6): 38.33, 48.06, 62.68, 111.55, 123.54, 125.99, 126.19, 126.52, 131.26, 130.82, 131.94, 134.08, 134.91, 134.95, 141.30, 159.1, 167.78, 179.93, 184.88; HR-MS (m/z, $[M+1]^+$): 429.1195 (calcd for $C_{23}H_{16}N_4O_5$, 428.1121)

Prediction of physicochemical parameters

The physicochemical properties of the synthesized compounds were predicted by the SwissADME calculator; based on the molecule's lipophilicity, hydrogen bonding,

Table 1 Physicochemical descriptors and absorption, distribution, metabolism and excretion properties of tested compounds; calculated by SwissADME.

Code	Chemical structure	MW[a]	LogP[b]	HBD[c]	HBA[d]	tPSA[e](Å^2)	Rotatable Bonds	Water solubility
Lawsone		174.15	1.21	1	3	54.37	0	Soluble
LME		188.18	1.43	0	3	43.37	1	Soluble
1		212.20	1.73	0	3	43.37	2	Soluble
4a		345.35	2.42	0	5	74.08	5	Poorly soluble
4b		371.39	2.97	0	5	74.08	6	Poorly soluble
4c		428.40	1.80	0	7	111.46	6	Poorly soluble

[a]MW=molecular weight, [b]log P=predicted octanol/water partition coefficient log P, [c]HBD=H-Bond Donors, [d]HBA=H-Bond Acceptors, [e]tPSA=topological polar surface area

rotatable bonds, and topological polar surface area compared to lawsone and Lawsone methyl ether (LME): as shown in Table 1.

Antimicrobial activity

Table 2 shows the MIC and MBC values of synthetic *N*-substituted 1,2,3-triazole (**4a-4c**) against *S. mutans, L. casei*, and *A. naeslundii.* Overall, the synthetic compounds **4a-4c** exhibited the most potent antimicrobial activity against *S. mutans* (MIC 6.25-50 µg/mL), followed by *A. naeslundii* (MIC 25-50 µg/mL), and were the least active against *L. casei.* However, MBC values could not be determined for all synthetic compounds tested.

The growth curves of *S. mutans, L. casei,* and *A. naeslundii* demonstrated that the presence of **4a-4c** at

Table 2 MIC and MBC values of synthetic lawsone derivatives containing *N*–substituted 1,2,3–triazole (**4a–4c**) and chlorhexidine gluconate against *S.mutans*, *L.casei,* and *A.naeslundii*.

Microorganisms	4a		4b		4c		LME[37]		CHX	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S.mutans	6.25	>100	6.25	>100	50	>100	1.56	>100	1.95	15.6
L.casei	>100	>100	>100	>100	>100	>100	50	>100	15.6	31.25
A.naeslundii	25	>100	6.25	>100	50	>100	6.25	100	1.95	3.9

MIC=minimum inhibitory concentration, MBC=minimum bactericidal concentration, CHX=chlorhexidine gluconate, LME=lawsone methyl ether, MIC and MBC are expressed in micrograms per milliliter (μg/mL)

the concentrations of 1/2 MICs prolonged the lag phase, reduced the slope of the exponential phase and decreased the peak absorbance (Figure 1).

Antibiofilm activity

Figure 2 shows that sub–MIC concentrations of all synthetic *N*–substituted 1,2,3–triazole (**4a–4c**) reduced the biofilm formation of *S. mutans* at 12 and 24 hours. The percentage of biofilm inhibition decreased with decreasing concentration; however, there was no statistically significant difference for each synthetic compound. At the concentrations of 1/2 MIC, the synthetic compounds **4a–4c** inhibited 12–hour biofilm formation by 96.87–98.71%. The inhibitory effect on 24–hour biofilm formation decreased to 69.71–81.55%. When the concentrations of all synthetic compounds were 1/4 MIC and 1/8 MIC, the 24–hour biofilm inhibition decreased to less than 40.0%.

Discussion

The 1,2,3–triazole scaffold was chosen in the design and synthesis of the target compounds because of its chemical and biological features. The scaffold has aromatic stability, resistance to acid–base hydrolysis, high dipole moment, and the ability to form H–bonds.[29] Furthermore, the triazole moiety readily associates with biological targets; such as deoxyribonucleic acid (DNA) via H–bonds and other noncovalent interactions, which improves the solubility and metabolic stability of the compound.[30] Copper–catalyzed azide–alkyne cycloaddition (CuAAC) is the most promising as well as widely utilized method for obtaining this product with high selectivity and yield.[31]

LME is a 1,4–naphthoquinone derived from lawsone with improved antifungal and antimicrobial activity. Anaissi–Afonso et al. suggested that the polar hydroxyl group in the lawsone structure hinders its penetration through the bacterial plasma membrane.[21] Sakunphueak and Panichayupakaranant proposed that the 2–methoxyl group in the LME structure promotes the absorption of LME molecules through the microbial cell membrane and increased its antimicrobial potency.[9] This was the motivation for the modification of the 2–hydroxyl group of the lawsone molecular structure to have more lipophilic moiety.

This present study demonstrated that the synthetic lawsone derivatives containing *N*–substituted 1,2,3–triazole possessed potent antibacterial activity against *S. mutans*, and moderate to potent activity against *A. naeslundii*. However, *L. casei* was resistant to these compounds. These synthetic lawsone derivatives contain two pharmacophores, quinone moiety in 1,4–naphthoquinone and triazole

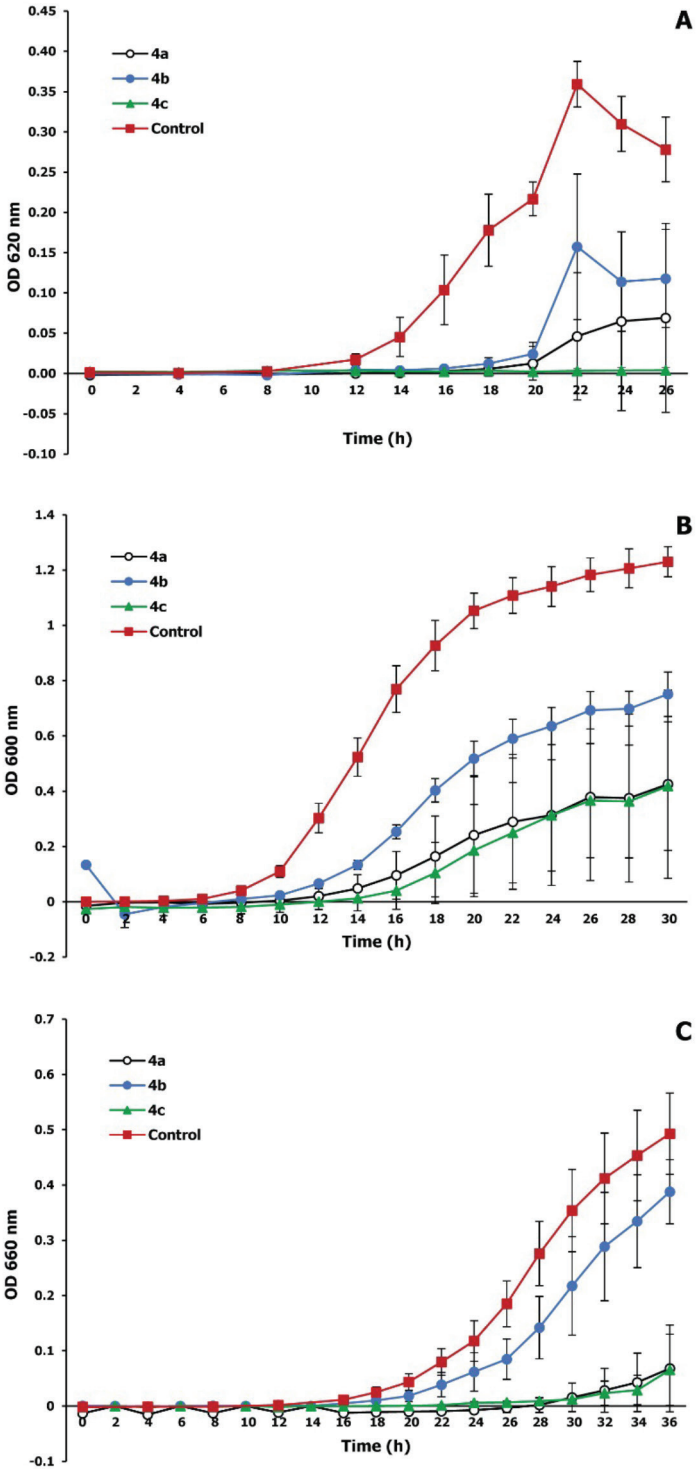
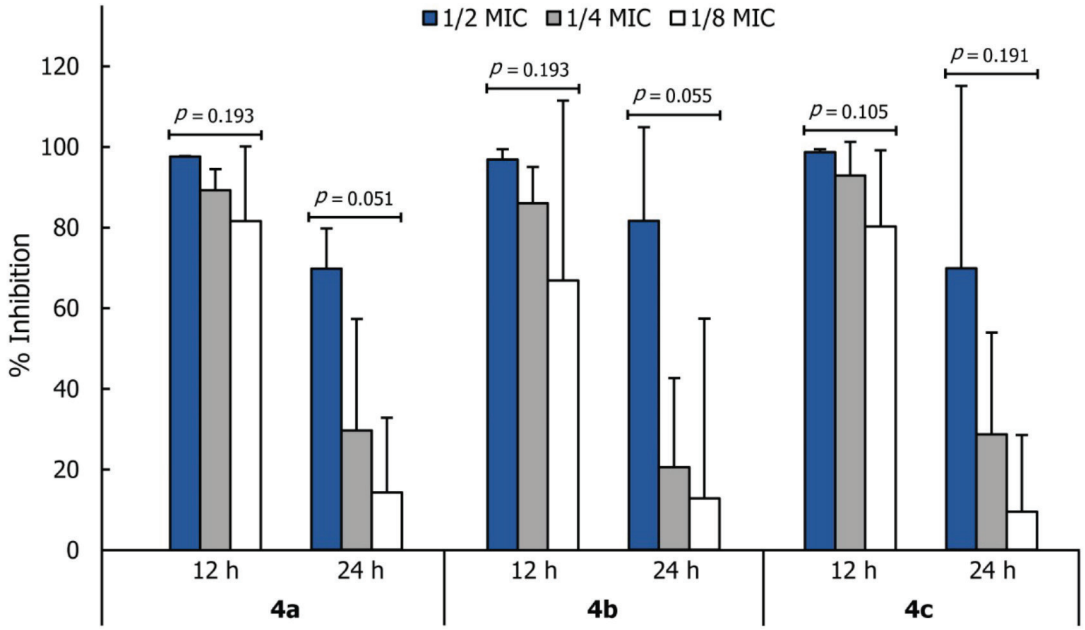


Figure 1 Growth curves of three bacterial strains with the presence of synthetic lawsone derivatives containing *N*–substituted 1,2,3–triazole (**4a**–**4c**) at the concentrations of 1⁄2 MICs; A. *S. mutans*; B. *L. casei*; C. *A. naeslundii*



MIC=minimum inhibitory concentration

Figure 2 Percentage of inhibition of 12-hour and 24-hour *S. mutans* biofilm formation, by synthetic lawsone derivatives, containing *N*-substituted 1,2,3-triazole (**4a**-**4c**) at concentrations of 1/2 MIC, 1/4 MIC, and 1/8 MIC

moiety that may exert enhanced antibacterial activity of their precursor, which is lawsone. Sakunphueak and Panichayupakaranant reported that the MIC of LME against *S. mutans* equaled 31.2 µg/mL, while the MIC of lawsone was higher than 500 µg/mL.[9] In this study, it was found that the MICs of synthetic lawsone derivatives containing *N*-substituted 1,2,3-triazole against S. mutans equaled 6.25 µg/mL for **4a** and **4b** and 50 µg/mL for **4c**. This may indicate that the structural modification of these synthetic lawsone derivatives possess improved antibacterial potency compared to their precursor. Since these compounds are more lipophilic, they may be able to penetrate bacterial cell membranes and reach the target of quinone moiety; which is a mitochondrial respiratory chain and bacterial biomolecules better than lawsone. The difference in oxygen requirements among tested strains may be the explanation for different antibacterial potency. *L. casei*, which is resistant to our synthetic lawsone derivatives, is an oxygen-tolerant anaerobes[32]; therefore, the lawsone derivatives did not affect the mitochondrial respiratory chain. While the other two strains are facultative anaerobes[33-34], which consume oxygen; resulting in ROS generation in mitochondria and subsequently cell destruction.

This study demonstrated that synthetic lawsone derivatives containing *N*-substituted 1,2,3-triazole have outstanding antibiofilm properties against *S. mutans*. Biofilm formation at 12th hour was reduced by more than 95.0% via lawsone derivatives at the concentration of 1/2 MIC. Although, antibiofilm activity of lawsone derivatives is dose- and time- dependent, even the inhibitory effect declined with time, and the percentages of biofilm formation of 1/2 MIC lawsone derivatives were still satisfying. The plausible

mechanisms of the inhibitory effect on *S. mutans* biofilm formation are: (i) these compounds inhibited the growth of *S. mutans* and thus affected the biomass of formed biofilm; (ii) they may promote bacterial aggregation, which results in less bacterial adherence; (iii) they may be able to inhibit the activity of glucosyltransferase, which is an essential enzyme of *S. mutans* for the synthesis of extracellular polysaccharides; the sticky matrix of biofilm.[35–36] However, the crystal violet assay used in this study could only measure the biofilm biomass. Further studies with other biofilm methods are required to explore the antibiofilm mechanism of these compounds.

Although the synthetic lawsone derivatives containing *N*–substituted 1,2,3–triazole in this study did not exhibit antibacterial potency comparable to the positive control (CHX), they still possess one valuable feature, this being antibiofilm activity against *S. mutans*. Such a specific effect on *S. mutans* biofilm formation, without any lethal effect on bacteria. Maybe even desirable and advantageous. This is because it means that these compounds selectively inhibit biofilm formation, a crucial process of dental caries formation, without disturbing the oral microbiome; including nonpathogenic microorganisms. Therefore, this may suggest that synthetic lawsone derivatives containing *N*–substituted 1,2,3–triazole are anticaries candidates. Hence this could be developed as a conjunctive therapy for dental caries prevention; via antibiofilm agents used and by combining them with fluoride or other anticaries agents.

Conclusion

This *in vitro* study showed that synthetic lawsone derivatives containing *N*–substituted 1,2,3–triazole have some inhibitory effect against *S. mutans* and *A. naeslundii,* which are dental caries pathogens. However, another significant attribute of these compounds is their outstanding antibiofilm properties against *S. mutans*. This suggests the potential use of synthetic lawsone derivatives containing *N*–substituted 1,2,3–triazole in dental caries prevention; as they could be developed as conjunctive anticaries agents. However, this is only the beginning step, as further studies are required to investigate for more potential benefits; such as other anticaries properties, and further structural modifications and to explore the mechanisms of these compounds.

Acknowledgement

The authors would like to thank the staff at the Dental Research Unit, Prince of Songkla University and Office of Scientific Instrument and Testing (OSIT), Prince of Songkla University for their assistance in conducting the lab testing process.

Funding sources

This research was supported by the National Science, Research, and Innovation Fund and Prince of Songkla University (Grant No. DEN6405026S), Faculty of Pharmaceutical Sciences (Grant No. PHA6304038S), and Graduate Research Fund, Prince of Songkla University.

Conflict of interest

The authors declare no conflict of interest in the publication of this paper.

References

1. Global Burden of Disease Study 2019 (GBD 2019) [homepage on the Internet]. Washington: Institute for Health Metrics and Evaluation; 2020. [cited 2022 May 29]. Available from: http://ghdx.healthdata.org/gbd-results-tool.
2. Yadav K, Prakash S. Dental caries: a microbiological approach. J Clin Infect Dis Pract 2017;02:118.
3. Tanner ACR, Kressirer CA, Rothmiller S, Johansson I, Chalmers NI. The caries microbiome: implications for reversing dysbiosis. Adv Dent Res 2018;29:78–85.

4. Jeon JG, Rosalen PL, Falsetta ML, Koo H. Natural products in caries research: current (limited) knowledge, challenges and future perspective. Caries Res 2011;45:243–63.
5. Howell A, Jr., Jordan HV, Georg LK, Pine L. Odontomyces viscosus, gen. nov., spec. nov., a filamentous microorganism isolated from periodontal plaque in hamsters. Sabouraudia 1965;4:65–8.
6. Caufield PW, Schon CN, Saraithong P, Li Y, Argimon S. Oral lactobacilli and dental caries: a model for niche adaptation in humans. J Dent Res 2015;94 (9 Suppl):110S–8S.
7. Panichayupakaranant P, Septama AW, Sinviratpong A. Synergistic activity of lawsone methyl ether in combination with some antibiotics and artocarpin against methicillin–resistant Staphylococcus aureus, Candida albicans, and Trychophyton rubrum. Chin Herb Med 2019;11:321–5.
8. Yang X, Summerhurst DK, Koval SF, Ficker C, Smith ML, Bernards MA. Isolation of an antimicrobial compound from Impatiens balsamina L. using bioassay–guided fractionation. Phytother Res 2001;15:676–80.
9. Sakunphueak A, Panichayupakaranant P. Comparison of antimicrobial activities of naphthoquinones from Impatiens balsamina. Nat Prod Res 2012;26:1119–24.
10. Lopez L, López L, Nery–Flores S, Flores N, Yesenia S, Silva Y, et al. Naphthoquinones: biological properties and synthesis of lawsone and derivatives. Vitae 2014;21:248–58.
11. Aneja B, Azam M, Alam S, Perwez A, Maguire R, Yadava U, et al. Natural product–based 1,2,3–triazole⁄sulfonate analogues as potential chemotherapeutic agents for bacterial infections. ACS Omega 2018;3:6912–30.
12. Nural Y, Ozdemir S, Doluca O, Demir B, Yalcin MS, Atabey H, et al. Synthesis, biological properties, and acid dissociation constant of novel naphthoquinone–triazole hybrids. Bioorg Chem 2020;105:104441.
13. Bunders C, Cavanagh J, Melander C. Flustramine inspired synthesis and biological evaluation of pyrroloindoline triazole amides as novel inhibitors of bacterial biofilms. Org Biomol Chem 2011;9:5476–81.
14. Minvielle MJ, Bunders CA, Melander C. Indole⁄triazole conjugates are selective inhibitors and inducers of bacterial biofilms. Med Chem Comm 2013;4:916–9.
15. Ballard TE, Richards JJ, Wolfe AL, Melander C. Synthesis and antibiofilm activity of a second–generation reverse–amide oroidin library: a structure–activity relationship study. Chemistry 2008;14:10745–61.
16. Rogers SA, Melander C. Construction and screening of a 2–aminoimidazole library identifies a small molecule capable of inhibiting and dispersing bacterial biofilms across order, class, and phylum. Angew Chem Int Ed Engl 2008;47:5229–31.
17. Huigens RW, 3rd, Rogers SA, Steinhauer AT, Melander C. Inhibition of Acinetobacter baumannii, Staphylococcus aureus and Pseudomonas aeruginosa biofilm formation with a class of TAGE–triazole conjugates. Org Biomol Chem 2009;7:794–802.
18. Nagender P, Malla Reddy G, Naresh Kumar R, Poornachandra Y, Ganesh Kumar C, Narsaiah B. Synthesis, cytotoxicity, antimicrobial and anti–biofilm activities of novel pyrazolo[3,4–b] pyridine and pyrimidine functionalized 1,2,3–triazole derivatives. Bioorg Med Chem Lett 2014;24:2905–8.
19. Linares D, Bottzeck O, Pereira O, Praud–Tabariès A, Blache Y. Designing 2–aminoimidazole alkaloids analogs with anti–biofilm activities: Structure–activities relationships of polysubstituted triazoles. Bioorg Med Chem Lett 2011;21:6751–5.
20. Praud–Tabaries A, Dombrowsky L, Bottzek O, Briand J–F, Blache Y. Synthesis of a polyprenyl–type library containing 1,4–disubstituted–1,2,3–triazoles with anti–biofilm activities against Pseudoalteromonas sp. Tetrahedron Lett 2009;50:1645–8.
21. Anaissi–Afonso L, Oramas–Royo S, Ayra–Plasencia J, Martín–Rodríguez P, García–Luis J, Lorenzo–Castrillejo I, et al. Lawsone, juglone, and β–lapachone derivatives with enhanced mitochondrial–based toxicity. ACS Chem Biol 2018;13:1950–7.
22. Petrat W, Wattanapiromsakul C, Nualnoi T, Sabri NH, Lee VS, Lomlim L. Cholinesterase inhibitory activity, kinetic and molecular docking studies of N–(1–substituted–1H–1,2,3–triazole–4–yl)–aralkylamide derivatives. Walailak J Sci Technol 2016;14:687–701.
23. Daina A, Michielin O, Zoete V. SwissADME: a free web tool to evaluate pharmacokinetics, drug–likeness and medicinal chemistry friendliness of small molecules. Sci Rep 2017;7:42717.
24. Potts RO, Guy RH. Predicting skin permeability. Pharm Res 1992;9:663–9.
25. Brookes ZLS, Bescos R, Belfield LA, Ali K, Roberts A. Current uses of chlorhexidine for management of oral disease: a narrative review. J Dent 2020;103:103497.
26. Wilkins JC, Homer KA, Beighton D. Analysis of streptococcus

mutans proteins modulated by culture under acidic conditions. Appl Environ Microbiol 2002;68:2382–90.

27. Dieterle ME, Fina Martin J, Durán R, et al. Characterization of prophages containing "evolved" Dit/Tal modules in the genome of Lactobacillus casei BL23. Appl Microbiol Biotechnol 2016;100:9201–15.
28. Kawashima J, Nakajo K, Washio J, Mayanagi G, Shimauchi H, Takahashi N. Fluoride-sensitivity of growth and acid production of oral actinomyces: comparison with oral streptococcus. Microbiol Immunol 2013;57:797–804.
29. Jiang X, Hao X, Jing L, Wu G, Kang D, Liu X, et al. Recent applications of click chemistry in drug discovery. Expert Opin Drug Discov 2019;14:779–89.
30. Xu Z, Zhao SJ, Liu Y. 1,2,3-Triazole-containing hybrids as potential anticancer agents: current developments, action mechanisms and structure-activity relationships. Eur J Med Chem 2019;183:111700.
31. Kolb HC, Finn MG, Sharpless KB. Click chemistry: diverse chemical function from a few good reactions. Angew Chem Int Ed Engl 2001;40:2004–21.
32. Zotta T, Parente E, Ricciardi A. Aerobic metabolism in the genus lactobacillus: impact on stress response and potential applications in the food industry. J Appl Microbiol 2017;122:857–69.
33. Ahn SJ, Wen ZT, Burne RA. Effects of oxygen on virulence traits of streptococcus mutans. J Bacteriol 2007;189:8519–27.
34. van der Hoeven JS, van den Kieboom CWA. Oxygen-dependent lactate utilization by Actinomyces viscosus and Actinomyces naeslundii. Oral Microbiol Immunol 1990;5:223–5.
35. He Z, Huang Z, Jiang W, Zhou W. Antimicrobial activity of cinnamaldehyde on streptococcus mutans biofilms. Front Microbiol 2019;10:2241.
36. Hasan S, Danishuddin M, Khan AU. Inhibitory effect of zingiber officinale towards Streptococcus mutans virulence and caries development: in vitro and in vivo studies. BMC Microbiol 2015;15:1.
37. Ratti P, Manuschai J, Kara J, Lomlim L, Naorungroj S. Effects of synthetic lawsone derivatives on streptococcus mutans biofilm formation. poster presented at: 35th annual scientific meeting, IADR-SEA Division; 2021 Dec 8–9; Hong Kong, China.