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Synthesis of benzoyl esters of β-amyrin and lupeol and evaluation of their antibiofilm and antidiabetic activities

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ABSTRACT

Diabetes as well as the enhanced microbial multidrug resistance resulting from biofilm formation, constitutes some of the major health problems around the world. Triterpenoids and their derivatives have been shown to have a great contribution in this domain. A small library of benzoyl esters of lupeol and *β*-amyrin was synthesized and their structures were characterized by electronic ionization mass spectrometry (EIMS). Their inhibitory potential on pathogenic bacteria biofilms, as well as their inhibitory action on α-amylase and β-glucosidase activities were evaluated. The mass fragmentation patterns from the EIMS data confirm the success of the reactions. The minimal inhibitory concentrations (MIC) varied from 250 to 1000 μg/mL in the antimicrobial activities. Biofilm inhibitory potential of the compounds on *S. aureus, E. coli* and *C. albicans* were performed at MIC and sub-MIC concentrations and the results showed concentration-dependent inhibition of biofilms. At MIC, the highest biofilm inhibition was exhibited by compound **7** on *S. aureus* (60.8 ± 3.2%), compound **3** on *E. coli* (60.5 ± 2.8%) and compound **8** on *C. albicans* (56.9 ± 2.5%). For all tested compounds, percentage inhibition of violacein production was 100% at MIC except for the starting compounds **1** and **2**. At 24.24 μg/mL the percentage of inhibition varied from 22.9 ± 1.2% to 42.1 ± 1.0% for α-amylase inhibition and at a concentration of 10 μg/mL the percentage of inhibition varied from 49.8 ± 0.3% to 69.3 ± 1.0% for β-glucosidase inhibition. The highest inhibition was shown by compounds **7** and **8** on α-amylase and β-glucosidase assays, respectively. The results show that introduction of benzoyl ester groups at C-3 of lupeol and β-amyrin considerably improves their antibiofilm and antidiabetic potentials.

Introduction

The great diversity of molecular targets and structural characteristics of natural compounds makes them the origin and development of new drugs, challenging organic chemists to develop innovative synthetic strategies inspired by the arsenal of natural products [1]. Total syntheses and chemical changes are the basis of research and development of innovative drugs obtained from chemically modified natural compounds with various functional groups that greatly improve their biological properties. [2]. The emerging need to modify the structures of natural products can be justified by the fact that organisms acquire multiple resistance to secondary metabolites produced by plants used for antimicrobial purposes, therefore new chemically modified compounds are needed to combat infectious diseases [2].The main goal of structural

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modification of natural products is to obtain new drugs [3]. Triterpenoids are suitable starting materials for chemical modifications and hemi-syntheses. Over 20,000 triterpenes are known to naturally occur in plants, especially the pentacyclic triterpenes which are one of the most important biologically active class of compounds widely distributed throughout the plant kingdom. β-amyrin and lupeol could be noted as representative members of the pentacyclic triterpene family [4,5]. Recently, there has been an increased interest in many studies about pentacyclic triterpenes concerning their therapeutic potential such as cardioprotective, hepatoprotective, gastro-protective, antidiabetic, antitumor, anti-inflammatory, antiviral, antimicrobial, analgesic, anti-parasitic, wound-healing effects etc of which hepatoprotective, anti-HIV, antitumor and anti-inflammatory activities. Some of this type of compounds are already marketed as therapeutic agents while some of their synthetic derivatives are undergoing clinical trials [4]. It has been reported also that some hemisynthetic triterpenoid derivatives, particularly the oleanane, ursane and lupane, are at the threshold of being incorporated to complement existing therapeutic approaches [6]. Propolis, especially the one from tropical and subtropical areas such as Cameroon, is a strong natural antibiotic and it is a good source of triterpenes because in this areas is rich in oleanane and lupane triterpenoids [7–13].

Due to the emergence and propagation of antimicrobial resistance and to the difficulties to develop new antimicrobial drugs, innovative strategies to control and combat bacterial infections are highly needed and natural and hemisynthetic pentacyclic triterpenoids presents a wide spectrum of applications in this domain [14]. Microbial infections caused by these multi-drug resistant bacteria pose a global health problem because some of these bacteria can mutate in the face of adversity causing drug-resistance towards traditional but also to new antimicrobial agents [15]. Therefore, the continuous search for new therapies capable of treating bacterial infections by disrupting bacterial cell-to-cell communication and bacterial biofilm formation is of great interest, these therapies can be developed with natural or synthetic products [16–18].

Diabetes mellitus is characterized by metabolic conditions involving hyperglycemia resulting either from insulin inefficiency or insulin secretion defects. Diabetes was estimated to affect over 177 million people worldwide in 2005 and this number is expected to attain 300 million patients by 2025 [19]. Cost-effective measures are prescribed to control type II diabetes risk such as healthy living style, consumption of antioxidant and green foods with anti-diabetic potential. In addition, there is a growing interest in finding new herbal antidiabetic medicines and herbal therapies [20,21]. Type 2 diabetes accounts for over 90% of cases of diabetes, and some of the drugs available for its treatment have many side effects, so new safer antidiabetic therapies are needed [22]. Patients with diabetes are severely affected by chronic wound infections and slow wound healing due to insulin treatment, which intensifies the formation of biofilm of pathogenic microorganisms by elevating the intracellular cyclic di-GMC levels [23]. For patients with diabetic foot ulcers, it is difficult to make the difference between colonization and infection with microorganism, because some alone non-pathogenic bacteria can interact symbiotically in a pathogenic biofilm and lead to a chronic infection that progresses further with other health complications [24]. Therefore, it is necessary to develop new molecules with therapeutic potential to prevent and treat complications of diabetes, such as biofilm formation or wound infections.

In this paper, benzoyl esters of β-amyrin and lupeol with antidiabetic and antibiotic potential have been synthesized and evaluated to see if newly obtained and characterized synthetic compounds have the same effect, greater or lesser than the two isolated natural compounds from propolis. In vitro and *in vivo* pharmacological studies are underway to obtain and test antidiabetic pharmaceutical formulations and also materials for the healing of wounds and prevention of wound infections, for patients with diabetes. Therefore, in the present research, preliminary studies have been performed to identify the influence of synthetic derivatives of lupeol and β-amyrin on two enzymes involved in type II diabetes but also their antimicrobial action.

Materials and methods

General information

In this study, a series of new benzoyl ester analogues of lupeol and β-amyrin have been successfully synthesized by introducing various acyl substituents at C-3 OH function, through esterification with benzoyl chlorides having a different number of methoxy groups on the phenyl ring. N, N-dimethyl-4-aminopyridine (DMAP) and N, N′-diisopropylcarbodiimide (DIC) were used as catalysts while the solvent was dichloromethane. The structures of the synthesized compounds were characterized using extensive EIMS (Electronic Ionization Mass Spectrum) data and their respective mass fragmentation patterns. The bioactivities of these compounds were investigated *in vitro*. All the reagents and solvents were of analytical grade and were used without additional purification. α-amylase A3403 Termamyl® (EC 3.2.1.1) from *Bacillus licheniformis*, β-glucosidase G0395 (EC 3.2.1.21) from almonds and acarbose A8980 were obtained from Sigma-Aldrich Chemical Company (St. Louis, USA) alongside 3,4,5-trimethoxybenzoyl chloride, p-methoxybenzoyl chloride, methoxybenzoyl chloride, DIC and DMAP. Lupeol and β-amyrin were isolated from Cameroonian propolis as described elsewhere [25]. Melting points (m.p.) were recorded on a Buchi M−560 melting point apparatus equipped with a Buchi M−569 sample loader. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra for lupeol and β-amyrin were recorded in deuterated chloroform, CDCl3 on a Bruker Avance-500. EIMS was performed on a JEOL MSRoute-600H mass spectrometer. Optical densities were measured on a Thermo Scientific Multiskan FC, Vantaa, Finland for antimicrobial and antibiofilm studies and on a Tecan Pro 200, Tecan Trading AG, Männedorf, Switzerland, multiwell plate reader for enzyme inhibitory assay.

Synthesis of benzoyl esters of lupeol and β-amyrin

Firstly, esters were prepared form lupeol and benzoyl chloride, p-methoxy benzoyl chloride and 3,4,5-trimethoxybenzoyl chloride. Equally, esters from β-amyrin and benzoyl chloride, p-methoxy benzoyl chloride and 3,4,5-trimethoxybenzoyl chloride were prepared. Finally, esterification of lupeol and β-amyrin was done using suitable benzoyl chlorides with N, N′-diisopropylcarbodiimide (DIC) as condensing agent and 4-dimethylaminopyridine (DMAP) as catalyst under mild conditions at room temperature in anhydrous dichloromethane [26]. The products obtained were purified on silica gel column chromatography with DCM: EtOAc (50:50) eluent. The general reaction is as given in Fig. 1 below, with the same reaction conditions (DIC, DMAP, CH_2Cl_2, room temperature, 24 h) but different R substituents on benzene ring (R_1, R_2, R_3 either H or CH_3O–).

Determination of MIC (Minimal inhibitory Concentration)

The bacterial and fungal strains *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 10239) and *Chromobacterium violaceum* (CV12472) were used.

The broth dilution method was applied to determine the MIC values [27]. The lowest concentration of test sample (compound) at which no microbial (bacterial and fungal) growth was visible was considered as the MIC. Mueller-Hinton Broth (MHB) was used as the medium and the microbial concentration used had a density of $5x10^5$ colony-forming units (CFU)/mL. Into 96-well microtiter plates, containing extracts at concentrations ions (2.0, 1.0, 0.5, 0.25, 0.125, 0.0625, 0.0312 mg/mL), 100 μL of microbial cell solutions were inoculated and incubated for 24 h at 37 °C, after which the MIC values were determined and recorded.

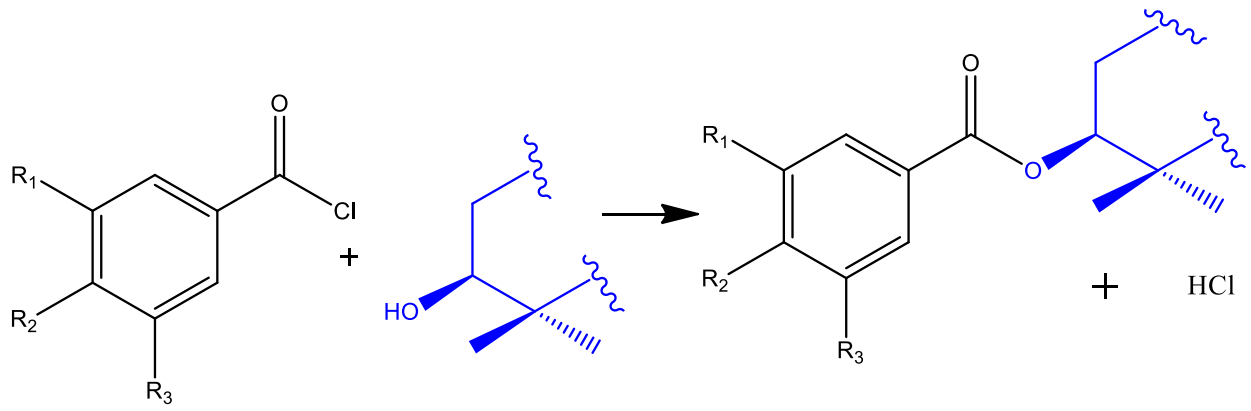

Fig. 1. General synthetic scheme.

Antibiofilm assay of compounds

The effect of the compounds at concentrations including 1, 1/2, 1/4, and 1/8 MIC on biofilm-forming ability of the tested microorganisms (*S. aureus, E. coli* and *C. albicans*) were evaluated by using the microplate biofilm assay [28,29]. Briefly, 1% of overnight cultures of isolates were added into 200 μL of fresh Tryptose-Soy Broth (TSB) supplemented with 0.25% glucose and cultivated in the presence and absence of the tested compounds without agitation for 48 h at 37 °C. The wells containing TSB + cells served as negative control. The two natural compounds (**1** and **2**) were compared with the 6 newly synthesized and characterized compounds, in which the natural compounds were considered positive control samples. After incubation, the wells were washed with water to remove planktonic bacteria. The remaining bacteria were subsequently stained with 0.1% crystal violet solution for 10 min at room temperature. Wells were washed once again to remove the crystal violet solution. A volume of 200 μL of 33% glacial acetic acid or ethanol was poured in wells. After shaking of wells, 125 μL of the solution from each well were transferred to a sterile tube and the volume was adjusted to 1 mL with distilled water. Finally, the optical density (OD) of each sample was measured at 550 nm (Thermo Scientific Multiskan FC, Vantaa, Finland). Percentage of inhibition of the tested compounds was calculated by using the formula:.

$$Biofilm\ inhibition\ (\%) = (OD_{control} - - OD_{sample}) / OD_{control} \cdot 100, \quad (1)$$

Violacein inhibition assay using C. violaceum CV12472

All the tested compounds were subjected to qualitative analysis to find their quorum sensing inhibition (QSI) potentials against *C. violaceum* CV12472 [14]. Overnight culture (10 μL) of *C. violaceum* (adjusted to 0.4 OD at 600 nm) was added into sterile microtiter plates containing 200 μL of LB broth and incubated in the presence and absence of sub-MIC concentrations of compounds. Broth containing *C. violaceum* ATCC 12,472 was used as a positive control. These plates were incubated at 30 °C for 24 h and observed for the reduction in violacein pigment production. The absorbance was read at 585 nm. Each experiment was done in triplicate and the percentage of violacein inhibition was calculated by following the formula:.

$$Violacein\ inhibition\ (\%) = (OD_{control} - - OD_{sample}) / OD_{control} \cdot 100, \quad (2)$$

In vitro α-amylase inhibition assay

The α-amylase inhibition assay was performed using the 3,5-dinitrosalicylic acid (DNS) method [30]. According to Ali et al. [31], a minimum concentration of 5 μg/mL of lupeol could inhibit α-amylase activity. So, in our study the percentage inhibition of α-amylase activity by the compounds was evaluated at a dose of 24.24 μg/mL. Each sample was dissolved in minimum amount of 10% DMSO and was further dissolved in phosphate buffer, pH = 6.8 ($(Na_2HPO_4/NaH_2PO_4$ (0.01 M), NaCl (6 mM)) to give stock concentrations of 200 μg/mL. A volume of 40 μL of α-amylase solution (4 mg/ml) was mixed with 80 μL of sample and was incubated for 20 min at 37 °C. Thereafter 140 μL of the starch solution (1% in water (w/v)) was added to each tube and incubated for 30 min. at 37 °C. The reaction was terminated by the addition of 400 μL of 1% DNS reagent and was boiled for 10 min in a water bath at 85–90 °C. The final concentration of each analyzed compound was 24.24 μg/mL. The two natural compounds (1 and 2) were compared with the 6 newly synthesized and characterized compounds, in which the natural compounds were considered positive control samples. The mixture was cooled to ambient temperature and was diluted with 800 μL buffer, and the absorbance was measured at 540 nm using a microplate reader (iTecan Microplate). The blank with 100% enzyme activity was prepared by replacing the samples with buffer. A blank reaction was similarly prepared using the sample in the absence of the enzyme solution. Acarbose was used as anti-amilase control standard (1–500 μg/mL). The α-amylase inhibitory activity was expressed as percent of inhibition and was calculated using the equation given below:.

$$\alpha - amylase\ inhibition(\%) = (OD_{control} - - OD_{sample}) / OD_{control} \cdot 100, \quad (3)$$

In vitro β -Glucosidase inhibition assay

β-Glucosidase inhibitory activity was determined as described by Chokki et al. [32]. Briefly, mixtures of 20 μL sodium phosphate buffer (pH 5.0), 20 μL of p-nitrophenyl-β-D-glucopyranoside (Sigma Chemical Co., 1 mg/mL) and 10 μL of the sample at a stock concentration of 200 μg/mL (dissolved in DMSO) were incubated in a 96-well plate at 37 °C for 10 min, followed by the addition of 10 μL enzyme solution β-Glucosidase from almonds (Sigma Chemical Co., 5 mg/mL), to each well, and incubation at 37 °C for 30 min. The final concentration of each inhibitor was 10 μg/mL. The reaction was terminated by adding 140 μL of sodium carbonate buffer pH = 10. Absorbance was determined at 410 nm using a microplate reader (iTecan Microplate). The control and blank were added 10 μL DMSO instead of the sample solution. The system without β-glucosidase was used as blank, and acarbose was used as positive control. The β-glucosidase inhibitory activity was expressed as the percentage of inhibition and calculated by the following equation:.

$$Inhibitory\ activity(\%) = 100 \cdot [1 - - (OD_{sample} - - OD_{blank/sample}) / (OD_{control-}OD_{blank/control})] \cdot 100, \quad (4)$$

where ODsample was the absorbance of the samples + enzyme + substrate; ODsample/blank was the absorbance of phosphate buffer + sample + substrate; ODcontrol was the absorbance of enzyme + substrate + solvent (DMSO); ODblank was the absorbance of substrate + solvent (DMSO).

Results

NMR data of substrate compounds (lupeol and β-amyrin)

The structures of starting compounds were confirmed by NMR analysis [25].

Lupeol: m.p. 170–172 ^{0}C. ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 38.8 (C-1), 27.5 (C-2), 79.0 (C-3), 39.9 (C-4), 55.3 (C-5), 19.3 (C-6), 34.2 (C-7), 41.1 (C-8), 50.5 (C-9), 37.2 (C-10), 21.2 (C-11), 25.3 (C-12), 38.5 (C-13), 42.8 (C-14), 27.2 (C-15), 35.6 (C-16), 43.0 (C-17), 48.3 (C-18), 47.8 (C-19), 150.9 (C-20), 30.1 (C-21), 40.3 (C-22), 28.4 (C-23), 16.1 (C-24), 16.0 (C-25), 15.6 (C-26), 14.5 (C-27), 18.1 (C-28), 109.3 (C-29), 20.2 (C-30). ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H ppm 1.98 (H-l), 2.20 (H-2), 3.19 (H-3), 0.69 (H-5), 1.45 (H-6), 1.40 (H-7), 1.17 (H-9), 1.40 (H-11), 1.88 (H-12), 1.67 (H-13), 1.75 (H-15), 1.38 (H-16), 1.35 (H-18), 2.38 (H-19), 1.83 (H-21), 1.42 (H-22), 1.04 (H-23), 0.97 (H-24), 1.40 (H-25), 0.84 (H-26), 0.79 (H-27), 1.26 (H-28), 4.56, 4.65 (H-29), 1.69 (H-30).

β-amyrin: m.p. 189–191 ^{0}C. ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 38.7 (C-1), 23.6 (C-2), 79.1 (C-3), 37.2 (C-4), 55.3 (C-5), 18.0 (C-6), 32.8 (C-7),41.5 (C-8), 47.6 (C-9), 36.8 (C-10), 28.1 (C-11), 121.8 (C-12), 145.3 (C-13), 42.1 (C-14), 26.6 (C-15), 31.1 (C-16), 40.8 (C-17), 50.5 (C-18), 28.1 (C-19), 33.7 (C-20), 39.6 (C-21), 39.7 (C-22), 28.1 (C-23), 16.7 (C-24), 15.6 (C-25), 16.8 (C-26), 23.2 (C-27), 17.5 (C-28), 18.7 (C-29), 21.3 (C-30). ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H ppm 1.91 (H-l), 1.85 (H-2), 3.24 (H-3), 0.88 (H-5), 1.54 (H-6), 1.57 (H-7), 1.67 (H-9), 1.94 (H-11), 5.18 (H-12), 2.17 (H-15), 1.94 (H-16), 1.94 (H-18), 1.38 (H-19), 1.44 (H-21), 2.06 (H-22), 0.80 (H-23), 0.91 (H-24), 0.77 (H-25), 0.94 (H-26), 1.15 (H-27), 0.81 (H-28), 1.08 (H-29), 0.84 (H-30).

Characterization of benzoyl esters

The various benzoyl esters were synthesized from β-amyrin (1) and lupeol (2) and the structures of the starting compounds and the derivatives obtained are shown in Fig. 2. The various synthesized derivatives are *β*-amyrin benzoyl ester (3), lupeol benzoyl ester (4), *β*-amyrin *p*-methoxybenzoyl ester (**5**), lupeol *p*-methoxybenzoyl ester (**6**), *β*-amyrin 3,4,5-trimethoxybenzoyl ester (**7**) and lupeol 3,4,5-trimethoxybenzoyl ester (**8**).

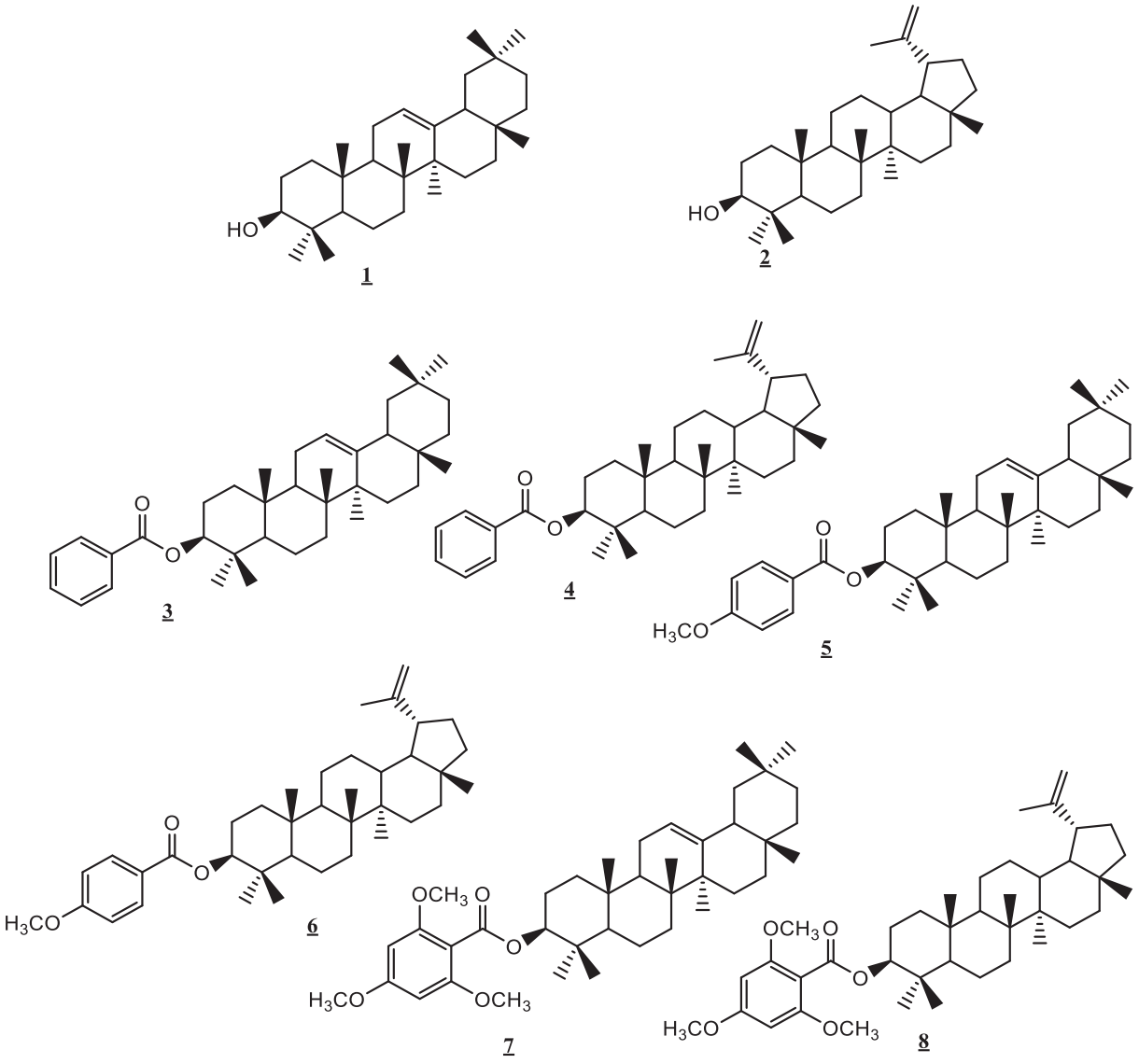

Fig. 2. Structures of substrate compounds and synthetic derivatives.

The EI-MS of compound **8** (code RBK in supplementary material) gave a molecular ion peak at *m/z* 620 confirming the theoretical molecular formula $C_{40}H_{60}O_5$ consistent with eleven degrees of unsaturation. The fragmentation pattern of compound **8** is presented in Fig. 3.

The EI-MS of compound **7** (code RBB in supplementary material) gave a molecular ion peak at *m/z* 620 confirming the theoretical molecular formula $C_{40}H_{60}O_5$ consistent with eleven degrees of unsaturation. The MS fragmentation pattern was characteristic of an oleanane derivative giving the base peak at *m/z* 218.2 and another important peak at *m/z* 402.2 resulting from the RDA fragmentation, as shown in Fig. 4.

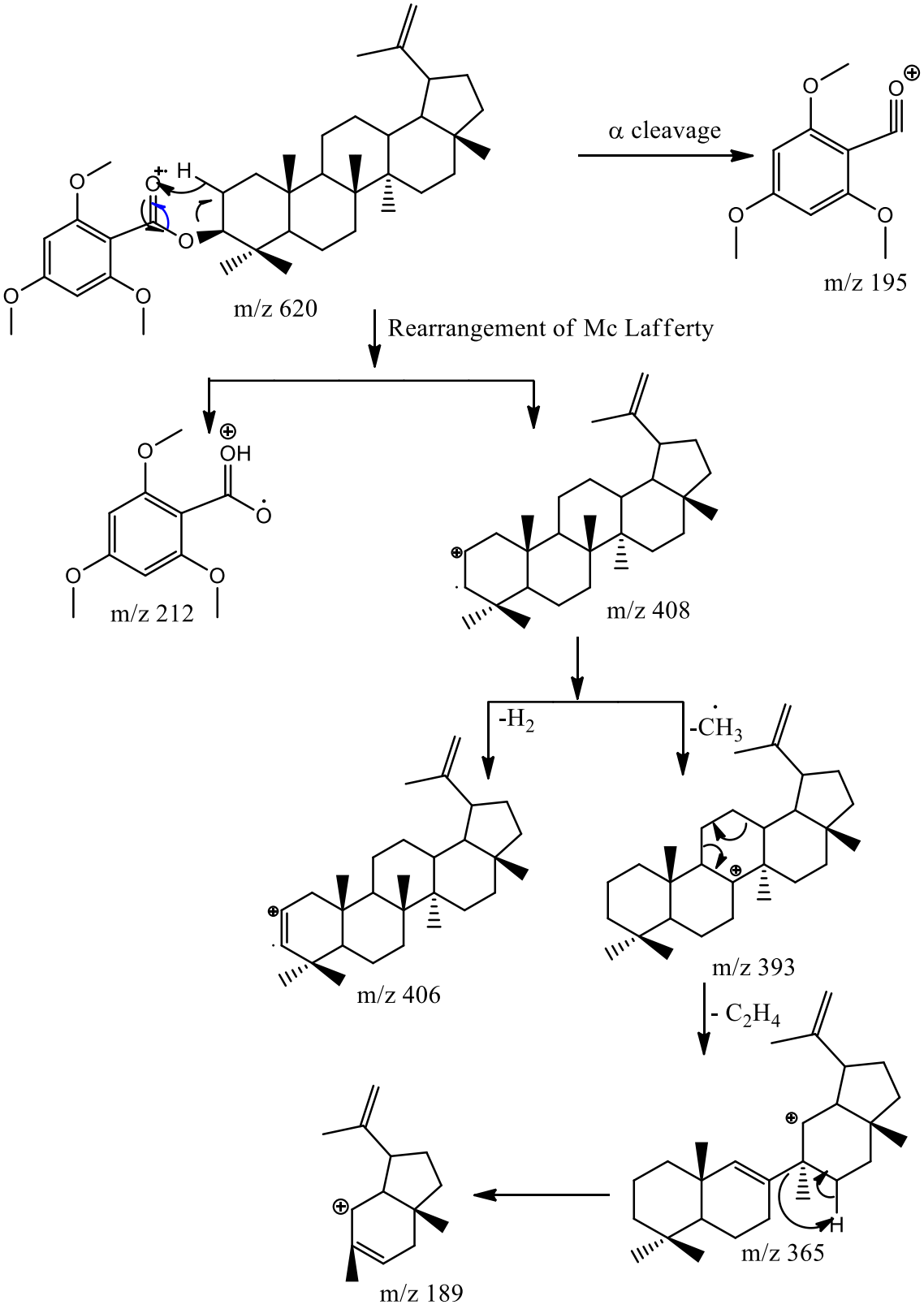

Fig. 3. Fragmentation pattern of compound **8.**

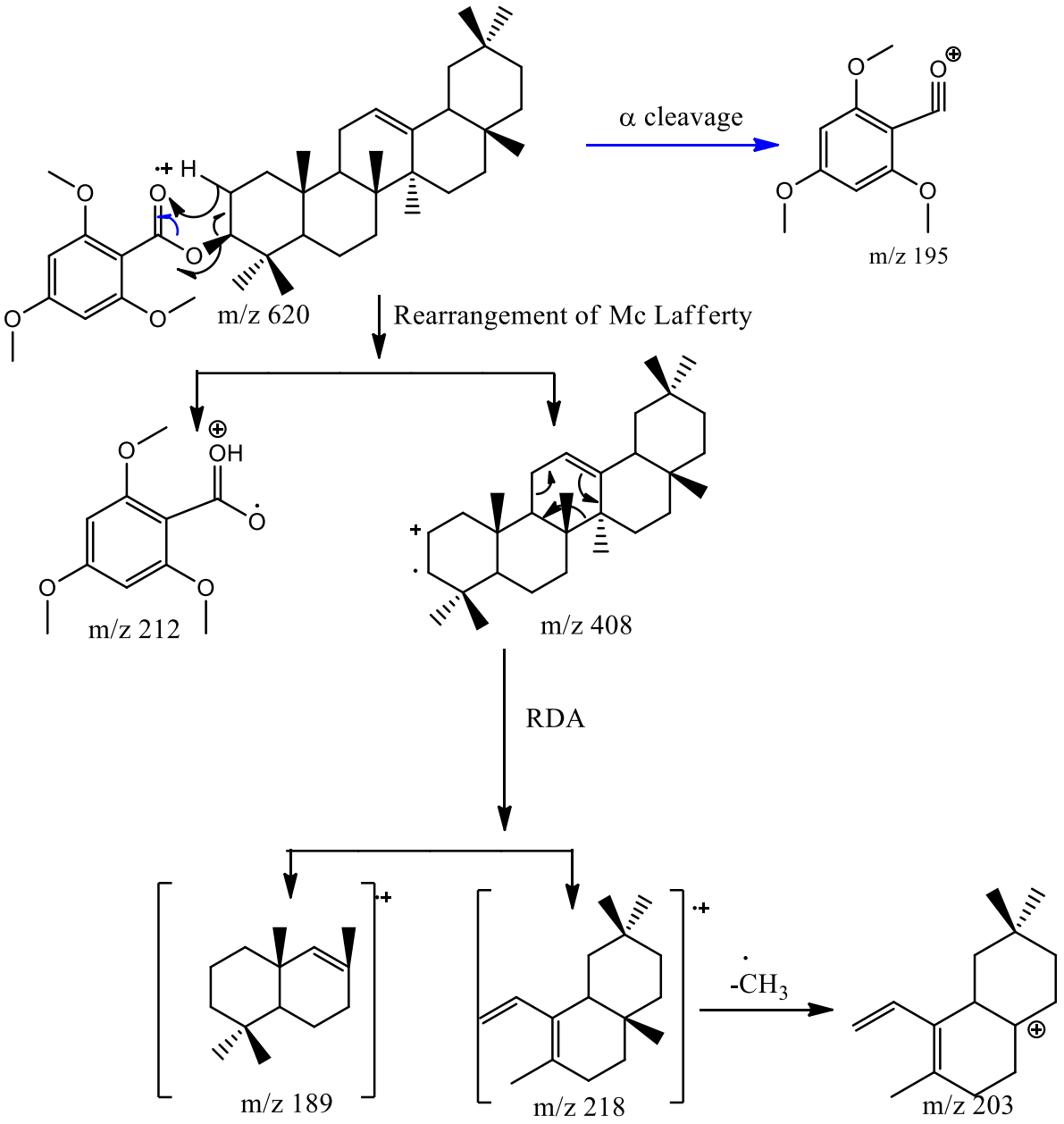

Fig. 4. Fragmentation pattern of compound **7**.

The EI-MS of compound **6** (code RBJ in supplementary material) gave a molecular ion peak at *m/z* 560 confirming the theoretical molecular formula $C_{38}H_{60}O_3$ consistent with eleven degrees of unsaturation. Other peaks at *m/z* 545.2 ($M-CH_3$) which subsequently loses the isopropenyl group to give the peak at *m/z* 504.2 and the characteristic peak of lupane type triterpenoids derivatives at *m/z* 189.2 are present. The fragmentation pattern of compound **6** is shown in Fig. 5.

The EI-MS of compound **5** (code RBA in supplementary material) showed a molecular ion peak at *m/z* 560 confirming the theoretical molecular formula $C_{38}H_{56}O_3$ consistent with eleven degrees of unsaturation. The characteristic base peak of oleanane type triterpenes appears at *m/z* 218.2 resulting from RDA fragmentation which subsequently loses a methyl group (218.2-CH_3) to give the peak at *m/z* 203 or an ethyl group to give the peak at *m/z* 189.2. The peak at *m/z* 135 results from the p-methoxybenzoyl fragment is also prominent. The key fragments from compound **5** are shown on Fig. 6.

The EI-MS of compound **4** (code RBP in supplementary material) indicated a molecular ion peak at *m/z* 530 confirming the theoretical molecular formula $C_{37}H_{54}O_2$ consistent with eleven degrees of unsaturation. Other peaks at *m/z* 189.1 characteristic of lupane type triterpenes which subsequently loses the isopropenyl group to give the prominent peak at *m/z* 147. The base peak at *m/z* 129.9 results from the fragmentation of the C_3-C_4 and C_3-C_2 bonds. The fragments besides other key fragments are shown in Fig. 7.

The EI-MS of compound 3 (code RBM in supplementary material) gave a molecular ion peak at *m/z* 530 confirming the theoretical molecular formula $C_{37}H_{54}O_2$ consistent with eleven degrees of unsaturation. Other peaks at *m/z* 218.1 resulting from RDA characteristic of oleanane type triterpenoids which subsequently loses a methyl group to give the prominent peak at *m/z* 203. The key fragmentation patterns of compound **3** are shown on Fig. 8.

Antimicrobial activity

β-amyrin and lupeol were tested for their antimicrobial activity alongside their semi-synthetic benzoyl ester derivatives. The results

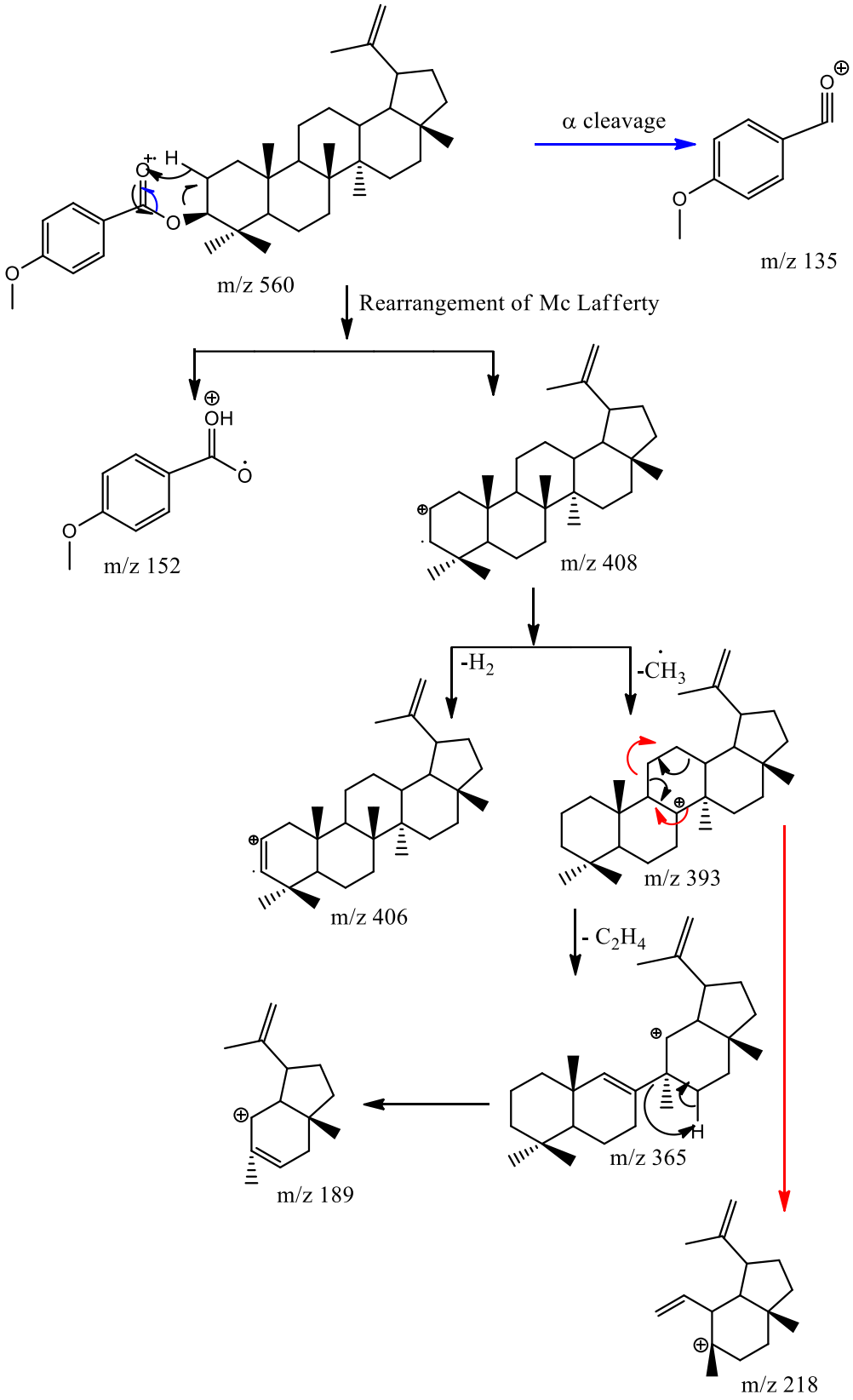

Fig. 5. Fragmentation pattern of compound **6.**

were given in terms of minimal inhibitory concentrations (MIC) as given in Table 1. The MIC values ranged from 250 μg/mL to 1000 μg/mL. Based on the MIC values, the most susceptible microorganism is *C. albicans*. The effects of compounds **1** and **2** were the same on all tested microorganisms and they were less active compared to their semi-synthetic counterparts. Amongst the semi-synthetic compounds, compounds **7** and **8** who had the highest amounts of methoxy-groups on the benzene ring were the most active. This could be due to the fact that

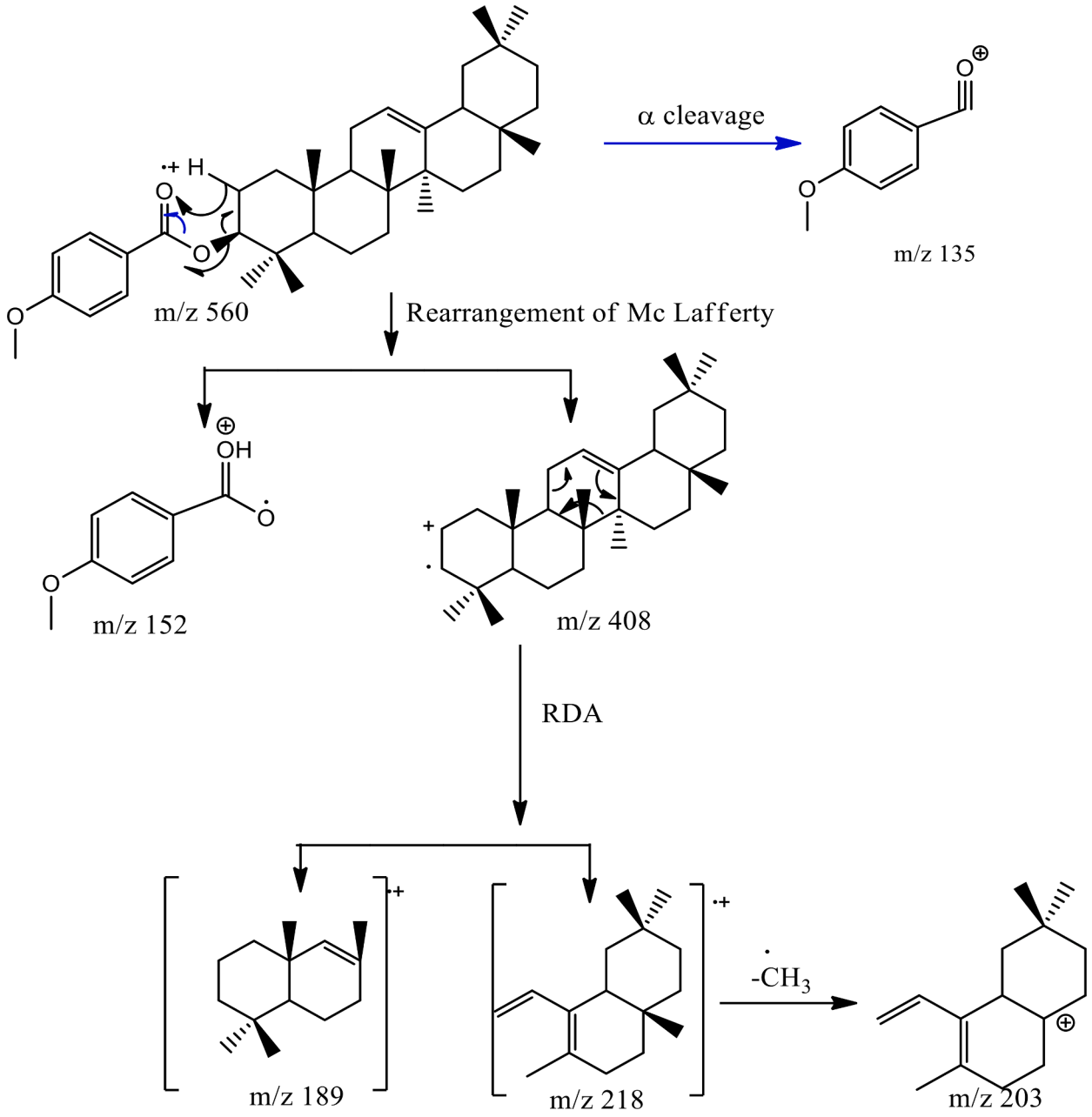

Fig. 6. Fragmentation pattern of compound **5**.

addition of methoxy substituents on the benzoyl ester derivatives caused an increase in the antimicrobial potential of the compounds.

Antibiofilm activity

Prior to antibiofilm assay, all the compounds were tested for antimicrobial activity as above, and their MIC values were determined. The biofilm inhibitory potential on *S. aureus, E. coli* and *C. albicans* of the compounds were performed at MIC and sub-MIC concentrations and the results reported in Table 2. This is because at weak concentrations, chances of development of resistant strains are low and equally the assay will be done under conditions which do not cause cell death or growth inhibition. On all microorganisms tested, the compounds inhibited the formation of biofilm in various percentages at MIC. On the Gram positive bacteria *S. aureus*, compound **7** had the highest biofilm inhibition which varied from 60.8 ± 3.2% (MIC) to 9.0 ± 0.1% (MIC/8) while compound **1** had the lowest biofilm activity of 25.8 ± 1.3% at MIC and could not inhibit biofilm at sub-MIC concentration. Only compounds **5**, **6**, **7** and **8** were able to inhibit biofilms at MIC/4, with compound **7** being the only one to inhibit biofilms at MIC/8 and no compound showed antibiofilm activity at MIC/16. On the Gram negative bacteria *E. coli*, all the compounds inhibited biofilm formation at MIC and MIC/2. Compound **3** showed the highest biofilm inhibition percentage which varied from 60.5 ± 2.8% (MIC) to 9.5 ± 0.4% (MIC/8) while compound **2** showed the lowest antibiofilm activity of 24.4 ± 1.0% at MIC and 5.7 ± 0.2% at MIC/2. Compounds **3**, **4**, **5** and **7** showed antibiofilm activity at MIC/4 while compounds **3** and **5** had potential to inhibit biofilms at MIC/8. On the yeast *C. albicans*, compound **8** showed highest antibiofilm activity ranging from 56.9 ± 2.5% at MIC to 8.1 ± 0.3% at MIC/16. All the compounds inhibited biofilm formation at MIC but only compound **8** inhibited biofilm formation at MIC/16. Compounds **6**, **7** and **8** had percentage inhibitions above 50% at MIC concentrations.

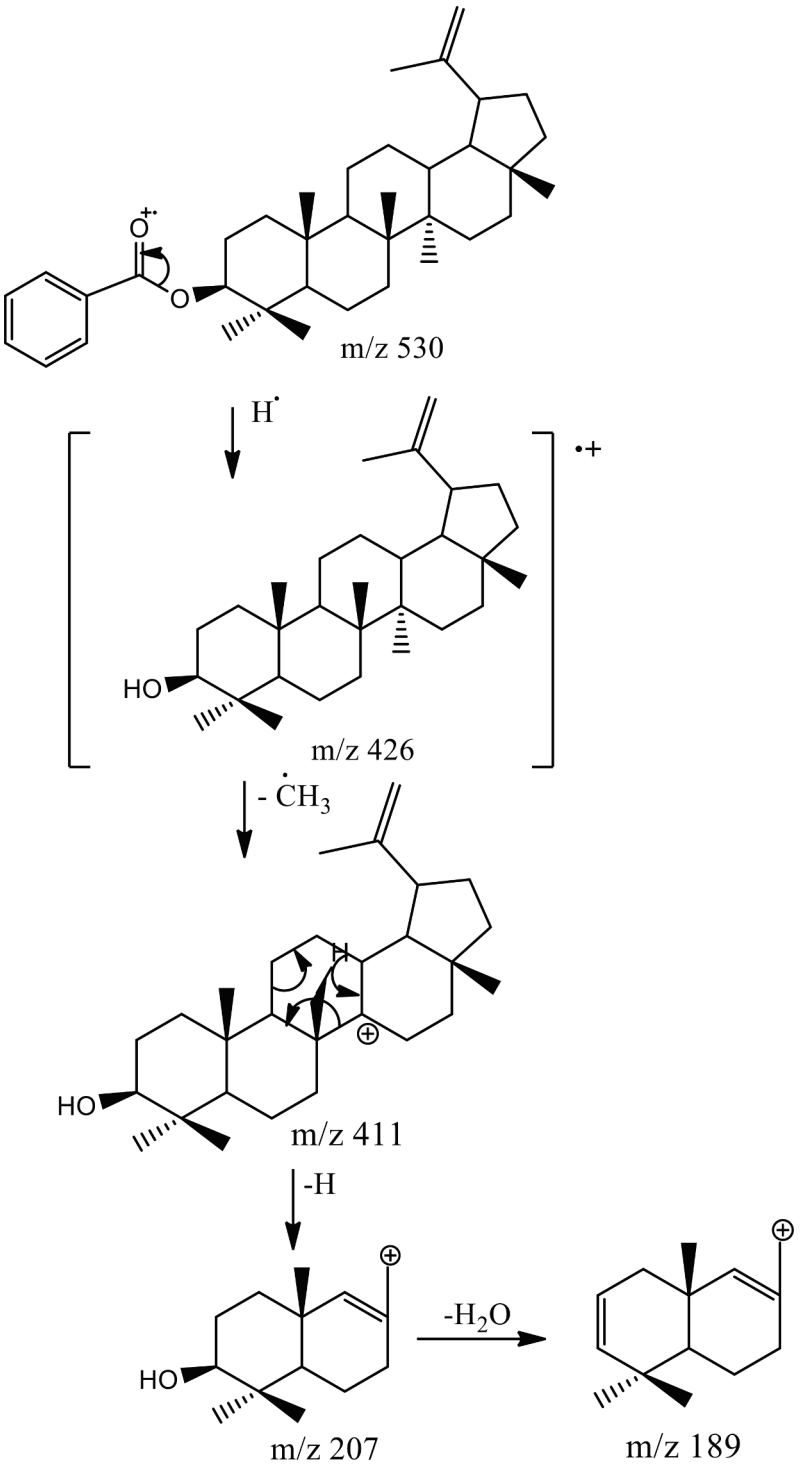

Fig. 7. Fragmentation pattern of compound **4.**

Inhibition of violacein production

The violacein production inhibition was performed using the indicator strain *C. violaceum* (CV12472) and is believed to usually result from either disruption of quorum-sensing (QS) signals or inhibition of cell growth. Prior to violacein inhibition, the MIC values for each sample were determined and the violacein inhibition assay was conducted with lesser concentrations to eliminate the hypotheses of growth inhibition. For all the tested compounds, percentage inhibitions of violacein production were 100% at MIC except for compounds **1** and **2** which had percentage inhibitions of 33.29 ± 1.23%, and 48.5 ± 0.89% respectively (Table 3). Compound **8** had the highest violacein inhibition of 100% at MIC and MIC/2 and was the only compound to show inhibition (15.31 ± 0.79%) at MIC/8 and no compound showed inhibition at MIC/16. (See Table 4).

Antidiabetic potential

The digestion of starch by α-amylase can be evaluated by using the dinitrosalicylic acid (DNS) assay, involving the reduction of one of the

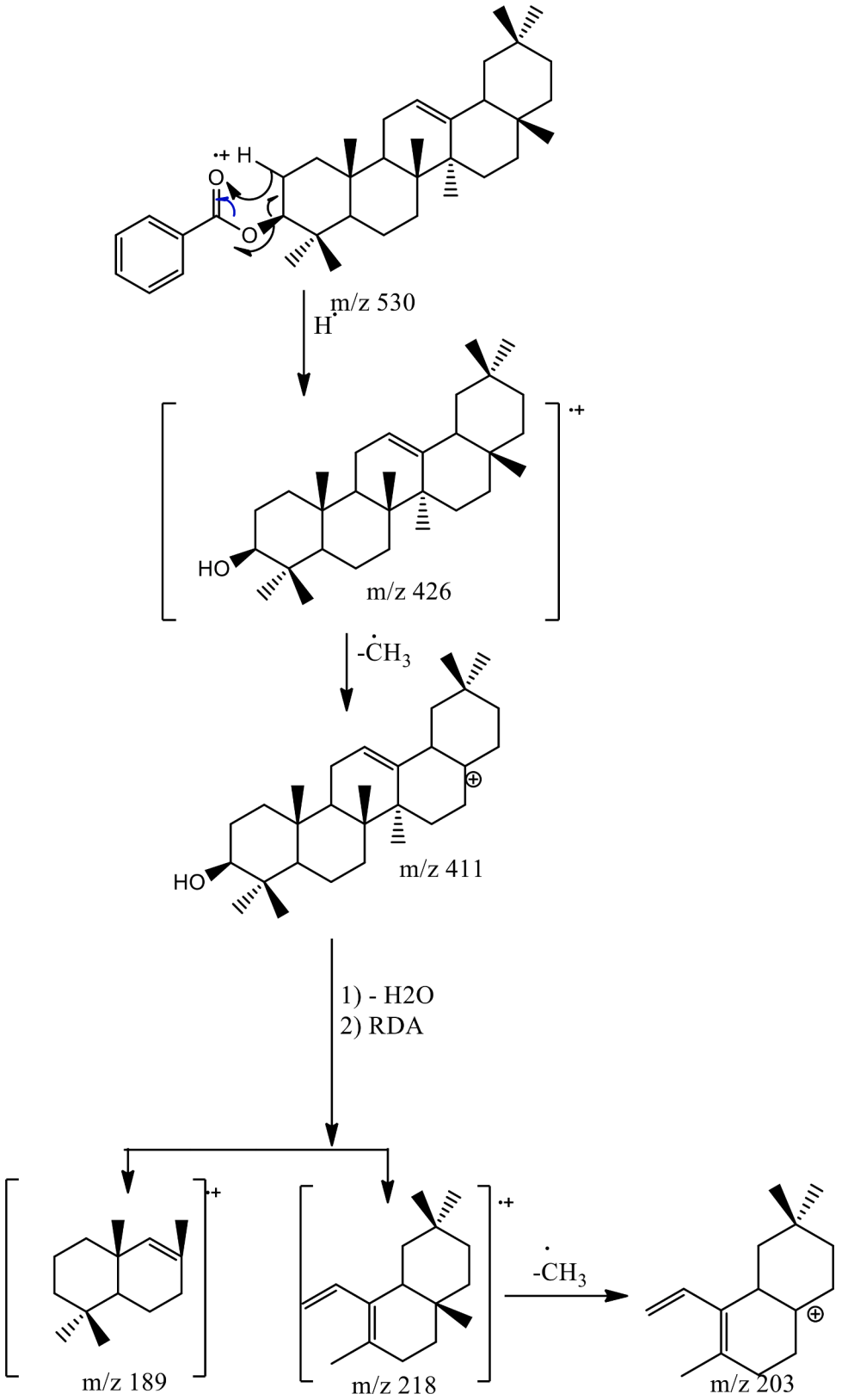

Fig. 8. Fragmentation pattern of compound 3.

nitro groups accompanied by a colour change which is interpreted as an indication the presence or absence of reducing sugars that supposedly results from on the hydrolysis of starch. It should be noted that the hydrolysis of starch into sugars can lead to hyperglycemia and diabetes and hence inhibiting the two enzymes responsible for starch hydrolysis, α-amylase and β-glucosidase can be beneficial. The percentage inhibition of α-amylase varied from 22.9 ± 1.2% to 42.1 ± 1.0%. The compound that showed the highest α-amylase inhibition is compound **7** while compound **1** showed the lowest α-amylase inhibition. For the β-glucosidase inhibitions, all the compounds had %inhibitions above

Table 1
Antimicrobial activity (MIC values μg/mL).

Microorganisms	Compounds 1	2	3	4	5	6	7	8
***S. aureus* ATCC 25,923**	1000	1000	1000	1000	500	1000	250	500
***E. coli* ATCC 25,922**	1000	1000	500	500	500	500	500	500
***C. albicans* ATCC 10,239**	1000	1000	500	500	250	500	250	250
***C. violaceum* CV12472**	1000	1000	1000	1000	1000	1000	1000	500

Table 2
Biofilm inhibition activity (percentage inhibitions) of the compounds 1–8.

Microorganism	Conc.	Biofilm inhibition (%) 1	2	3	4	5	6	7	8
	MIC	25.8 ± 1.3	39.5 ± 1.2	27.4 ± 1.4	34.2 ± 1.9	38.0 ± 1.3	37.8 ± 1.9	60.8 ± 3.2	38.2 ± 1.8
	MIC/2	–	24.5 ± 0.7	10.9 ± 0.3	15.7 ± 0.5	29.2 ± 1.1	28.2 ± 1.2	35.3 ± 1.1	32.3 ± 1.4
S. aureus	MIC/4	–	–	–	–	14.6 ± 0.3	12.0 ± 0.4	20.5 ± 0.5	10.7 ± 0.5
	MIC/8	–	–	–	–	–	–	9.0 ± 0.1	–
	MIC/16	–	–	–	–	–	–	–	–
	MIC	37.4 ± 1.52	24.4 ± 1.0	60.5 ± 2.8	52.3 ± 2.1	48.6 ± 1.8	35.0 ± 1.2	27.7 ± 1.8	43.0 ± 1.5
	MIC/2	18.0 ± 0.2	5.7 ± 0.2	45.2 ± 2.1	43.5 ± 0.6	42.1 ± 1.0	10.4 ± 0.5	17.1 ± 0.7	22.7 ± 0.6
E. coli	MIC/4	–	–	37.9 ± 1.3	24.3 ± 0.2	30.0 ± 0.6	–	10.2 ± 0.1	–
	MIC/8	–	–	9.5 ± 0.4	–	8.4 ± 0.2	–	–	–
	MIC/16	–	–	–	–	–	–	–	–
	MIC	19.7 ± 0.7	17.6 ± 1.0	26.9 ± 1.1	40.8 ± 1.5	44.8 ± 1.4	52.8 ± 3.1	54.5 ± 2.1	56.9 ± 2.5
	MIC/2	–	6.0 ± 0.5	7.5 ± 0.2	18.9 ± 0,9	35.2 ± 1.1	31.6 ± 1.8	38.8 ± 1.1	43.4 ± 1.9
C. albicans	MIC/4	–	–	–	6.1 ± 0.1	26.5 ± 0.9	23.2 ± 1.1	35.0 ± 0.3	38.6 ± 1.1
	MIC/8	–	–	–	–	15.0 ± 0.5	11.0 ± 0.6	14.8 ± 0.0	24.5 ± 0.9
	MIC/16	–	–	–	–	–	–	–	8.1 ± 0.3

Values represent the means ± SEM of three parallel measurements.
-: No activity.

Table 3
Percentage inhibition of violacein production on *C. violaceum* 12,472.

Conc.	Violacein pigment inhibition (% inh.) 1	2	3	4	5	6	7	8
MIC	33.29 ± 1.23	48.5 ± 0.89	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
MIC/2	15.95 ± 0.72	13.6 ± 0.21	17.48 ± 0.65	25.45 ± 0.96	17.61 ± 0.56	46.99 ± 1.47	47.8 ± 1.23	100 ± 0
MIC/4	–	–	9.81 ± 0.11	6.39 ± 0.32	11.43 ± 0.23	15.13 ± 0.21	18.13 ± 0.81	31.4 ± 1.25
MIC/8	–	–	–	–	–	–	–	15.31 ± 0.79
MIC/16	–	–	–	–	–	–	–	–

Values represent the means ± SEM of three parallel measurements.
-: No activity.

Table 4
Antidiabetic potential (percentage inhibition) of each compound at a concentration of 24.24 μg/mL in α-amylase assay, and 10 μg/mL in β-glucosidase assay.

Sample	α-amylase	β-glucosidase
1	22.9 ± 1.2	49.8 ± 0.3
2	24.3 ± 0.5	51.9 ± 0.5
3	25.7 ± 0.3	56.1 ± 1.0
4	29.1 ± 2.0	58.2 ± 0.5
5	32.0 ± 0.8	67.6 ± 0.7
6	34.3 ± 1.7	69.0 ± 1.1
7	42.1 ± 1.0	68.1 ± 0.2
8	40.6 ± 1.5	69.3 ± 1.0
Acarbose	36.2 ± 0.14 μg/mL	No inhibition [1–1000 μg/mL]

Values represent the means ± SEM of three parallel measurements.

50% at a concentration of 10 μg/mL except compound 1 though its inhibition was almost 50% (49.8 ± 0.3%). The highest %inhibition of β-glucosidase was exhibited by compound **8** (69.3 ± 1.0%). The percentage inhibitions of β-glucosidase were not much different amongst the compounds as it varied from 49.8 ± 0.3% to 69.3 ± 1.0%.

Discussion

Plants synthesize secondary metabolites for their own purposes and these compounds might require molecular and structural modifications so as to improve their biological activities or satisfy specific drug criteria. Lupeol and β-amyrin are the most ubiquitous and most important of the pentacyclic triterpenes found in medicinal plants and they have been proven to have biological activities alongside their synthetic derivatives [4]. These compounds were used as starting material for the synthesis of their benzoyl ester derivatives. The success of the synthetic procedure was evidenced by the electronic ionization mass spectrum (EIMS) of each of the compounds by comparing the expected molecular mass with the experimental or obtained molecular mass. The structures of the semi-synthetic compounds were confirmed by their detailed fragmentation patterns in conformity with the reported fragmented routes [33–36], as shown in Figs. 2 to 8 above. The key fragments as well as the molecular ions were used to unambiguously confirm the structures of the compounds. Fragments obtained from α-cleavage of the carbonyl function as well as Mc Lafferty rearrangements were prominent, as shown in Figs. 2-8. Also retro Diels-Alder rearrangements and other plausible fragment ions were important and accounted for important fragments.

Terpene esters of benzoic acids isolated from propolis have been

shown to possess good antimicrobial activities against Gram positive bacteria [37] and therefore, the antimicrobial activity of the synthesized triterpene benzoyl esters is justified. However, such compounds have not been evaluated for their antibiofilm activities previously, therefore, the antibiofilm activity exhibited by these compounds show that they could be potent compounds that can be used to remedy the severity of infections and resistance towards conventional antibiotics which may only kill planktonic bacteria while sessile bacteria colonies within biofilms will remain unaffected. This aspect is essential in the treatment of patients with diabetes whose wounds develop very serious pathogenic infections that progress further with other complications, such as superinfections, multidrug resistant microorganism and slow wound healing [24,38–41]. Microorganisms including bacteria, viruses, fungi, yeasts, protozoa and algae can adhere to each other and to surfaces thereby forming biofilms and these biofilms are ubiquitous [42]. The presence of antibiotics, starvation, immunological defense systems of host and other adverse conditions causes most pathogenic microorganisms to form protective coatings around self-organized and three-dimensional communities called biofilms, which accounts for the persistence and severity of infections [29,39]. Most of the existing antibiotics inhibit only the activities of planktonic bacteria that are not yet within established biofilms, while those that are already in biofilms can resist and will consequently cause reinfection through controlled processes by quorum sensing. Therefore, these compounds can be beneficial as they were shown to be able to inhibit biofilm formation. Disruption of microbial biofilms can eliminate resistance to drugs which is a serious health threat [43]. From the structures of the compounds, it could be seen that the more numerous the methoxy groups on the benzene ring of the benzoyl moiety, the greater the antibiofilm activity of the synthesized compound. Biofilm-associated resistance is caused by many factors, including the physiological state of cells and their population, as well as the physical structure of the biofilm whose matrix contains exopolysaccharides and extracellular DNA acting as a barrier to the diffusion and penetration of antibiotics into biofilms [44].

Diabetes mellitus (DM) is one of the major metabolic illnesses worldwide and is due to the inefficiency of insulin production by the pancreas or inefficiency of the body to use the insulin it produces [45,46]. Drugs such as metformin, bile acid sequestrant, dopamine agonist, dipeptidyl peptidase IV, meglitinides, sulfonylureas, sodium-glucose transport protein inhibitors, thiazolidinediones, and α-glucosidase inhibitors are usually employed to manage diabetes by lowering blood glucose levels [46,47]. There is a continuous search for new drugs that can treat diabetes and many natural products are being investigated for this purpose, including natural triterpenoids and their derivatives which seem to possess promising antidiabetic properties [48]. The two natural compounds **1** and **2** and their synthetic derivatives **3**–**8** are triterpenoids are therefore potential inhibitors of α-amylases and β-glucosidases (Fig. 9). The mentioned compounds inhibited these two enzymes and this is an indication that they can be applied in reducing the blood glucose levels and therefore in treating type 2 diabetes. The enzymes, α-amylase and β-glucosidase, are responsible for the hydrolysis of oligosaccharides into monosaccharide's which, if absorbed, will lead to increased blood sugar levels. Decreasing postprandial glucose levels is a strategy to remedy type 2 diabetic condition and could be achieved by inhibiting α-amylases and β-glucosidases, which causes a mechanism of delayed sugars absorbtion [49]. Although the mechanism of action of these triterpenes were not investigated, previous studies have shown antidiabetic activities of isolated triterpenoids and have been shown to regulate total cholesterol and triglycerides levels, control body weight, prevent pancreatic β-cell function, improve insulin sensitivity and glucose homeostasis, exert an effect on biosynthesis, secretion and signaling of insulin [48,50]. Plant-derived triterpenoids are considered safe and effective in the management of diabetes, and hence these compounds could have the added advantage of being able to reduce diabetic risks and ensure non-toxicity. Pentacyclic triterpenoid derivatives have been shown to have increased enzyme inhibitory activity against α -glucosidases *in vitro*, with a higher potency than ursolic acid and acarbose, especially when derivatized on C-2 as illustrated using ursolic acid [25]. This observation corroborates with our results, in which we found that the derivatization at C-3 position by introducing the benzoyl group increased the inhibitory activity on both α-amylase and β-glucosidase. However, these activities have also increased with the increase in the number of methoxy groups on the phenyl ring, thereby confirming that it is a suitable approach for ameliorating the antidiabetic potential of triterpene compounds by introducing acyl groups at the C-3 position. Of the 6 newly tested compounds, all caused a significant inhibition of the two enzymes when compared with the starting compounds, suggesting that the chemical modification of β-amyrin and lupeol could be important to obtain newly α-amylase and β-glucosidase inhibitory compounds. Subsequent molecular modeling

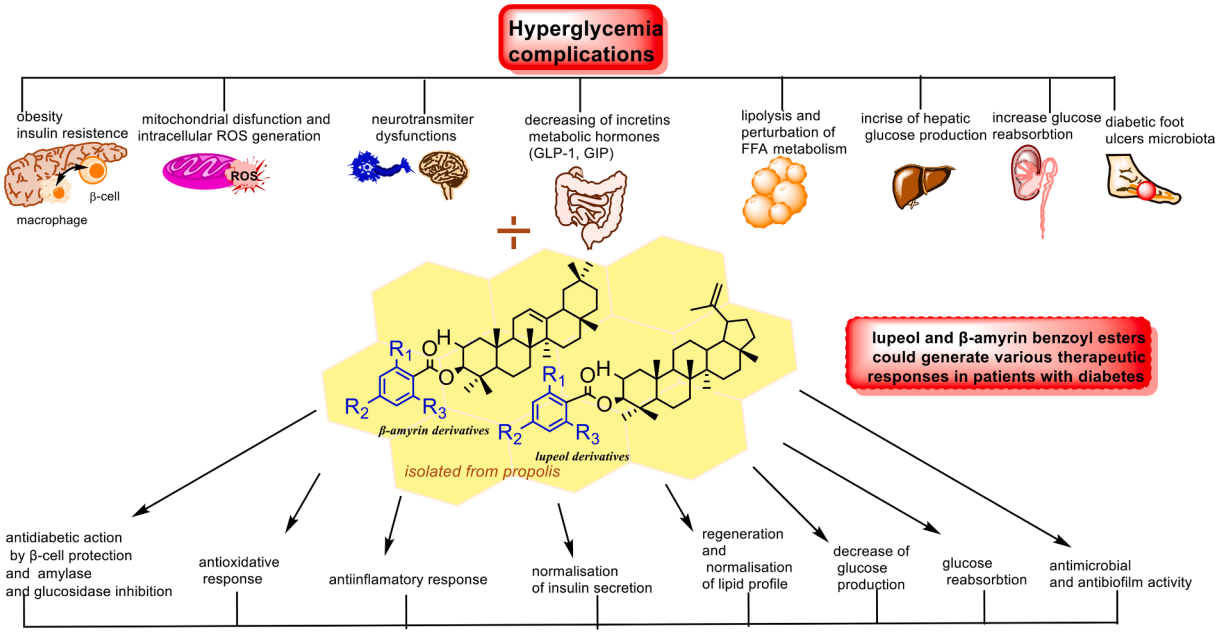

Fig. 9. Various mechanisms of actions of β-amyrin and lupeol triterpenoids for their antidiabetic activity.

studies will be conducted in future studies in order to better understand their mechanism of action. It is also interesting to mention that these derivatizations that increase the antidiabetic potential are not limited to acyl group or esterification, as some authors have mentioned an increased activity of the synthesized lupane, oleanane, ursane and dammarane triterpenoids derivatives on α-glucosidase by introducing different groups at C-2 and C-3 positions [51].

Conclusions

Triterpenoids are a class of compounds with high bioactive potential, including antimicrobial and antidiabetic properties. In this study, 6 new benzoyl esters were synthesized by esterification and characterized by EIMS. Their antidiabetic and antibiofilm potentials were evaluated. The results indicate that tritepenes and the semi-synthetic derivatives, such as their benzyl esters, could be potent α-amylase and β-glucosidase inhibitory activities that indicate that they could be used in the control and treatment of type 2 diabetes mellitus They also exhibited promising inhibition of bacterial biofilms formation and stands up the potential application of triterpenoids and their derivatives in the management of microbial resistance caused by biofilm formation in diabetic foot ulcer infections. These significant investigations highlight the promising applications of the analyzed compounds as pharmaceutical antidiabetic and antimicrobial formulations. Furthermore, these antimicrobial ingredients could be used for the surfaces sanitation of anti-infective medical devices, in antiseptics, as antibiotic compounds for other medicinal and industrial uses, as preservatives or disinfectants, but also as adjuvant compounds in the treatment of diabetes and its complications.

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Alfred Ngenge Tamfu: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Aristide Mfifen Munvera:** Investigation, Writing – original draft. **Andreea Veronica Dediu Botezatu:** Investigation, Writing – original draft. **Emmanuel Talla:** Visualization, Supervision. **Ozgur Ceylan:** Conceptualization, Methodology, Resources, Writing – review & editing. **Maurice Tagatsing Fotsing:** Writing – original draft, Visualization. **Joseph Tanyi Mbafor:** Visualization, Supervision. **Farzana Shaheen:** Resources, Visualization, Supervision. **Rodica Mihaela Dinica:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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