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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and antimicrobial activity of novel coumarin derivatives from 4-methylumbelliferone

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Abstract Considering the potential interest of heterocyclic compounds, the aim of the present study is to synthesize new coumarin derivatives, to provide their full chemical characterization and to evaluate their antimicrobial activities. The reaction of ethyl 2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy) acetate **2** with sodium hydroxide afforded the corresponding 2-(2-oxo-4-methyl-2*H*-chromen-7-yloxy) acetic acid **3** which was esterified using a series of alcohols in the presence of iodine to yield a new series of coumarin esters **4a–j**. On the other hand, treatment of the key intermediate **2** with an aqueous solution of hydrazine in ethanol at reflux gave the corresponding hydrazide **5** which further converted into coumarin derivatives **6a–f** and **7a–c** by condensation with a series of aromatic aldehydes and cyclic anhydrides, respectively. The synthesized compounds were completely characterized by ^{1}H NMR, ^{13}C NMR, IR and HRMS. The antibacterial and antifungal activities of the new synthesized compounds were evaluated using the disc diffusion method and seemed to be significant.

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Keywords
Ethyl 2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy) acetate · Coumarin esters · Coumarin hydrazides · Antifungal activity · Antibacterial activity

Introduction

Heterocyclic compounds are indispensable structural units that are useful in medicinal chemistry and valuable as synthetic organic blocks. In various classes of natural heterocyclic compounds of biological interest, the coumarin moiety is commonly present (Wu *et al.,* 2009). They display interesting biological and pharmacological profiles, such as the antibacterial (Al-Amiery *et al.,* 2011a), cytotoxic (Al-Amiery *et al.,* 2011a), antioxidant (Al-Amiery *et al.,* 2011a), antifungal (Al-Amiery *et al.,* 2012), anticoagulant (Manolov and Danchev, 1995), anti-inflammatory (Emmanuel-Giota *et al.,* 2001), antitumor and anti-HIV (Harvey *et al.,* 1988; Kostova *et al.,* 2006) activities. In addition, these compounds have varied bioactivities and applications in cosmetics, pharmaceuticals, food, flavoring and agrochemicals (Borges *et al.,* 2005; Kabalka *et al.,* 2005; Morimoto *et al.,* 2003; Srinivasan and Rampally, 2004).

Since the coumarin nucleus is associated with these diverse biological and pharmacological activities, several methods for the preparation of coumarin-based compounds have been reported (Gammonn *et al.,* 2005; Messaoudi *et al.,* 2010; Santana *et al.,* 2006). Particularly, the use of 4-methylumbelliferone as a precursor represents a useful synthetic method for the preparation of such compounds (Ganesh *et al.,* 2010; Kumar *et al.,* 2011a; Ramesh *et al.,* 2008).

Our research has been devoted to the development of a new class of heterocyclic systems which incorporate the

coumarin moiety with the hope that they may be biologically active.

Herein, we report here the synthesis of ethyl 2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy) acetate **2** and its use as a building block in the synthesis of some new coumarin-based derivatives **4–7**. Their antimicrobial activities were evaluated, and the structure–activity relationship was also discussed.

Our approach to the target coumarin derivatives was firstly started by the synthesis of product **2** via condensation reaction of equimolar amount of 4-methylumbelliferone, ethyl chloroacetate and anhydrous K_2CO_3 in dry DMF (Al-Amiery *et al.,* 2011b). Then, compound **2** was converted into the 2-(2-oxo-4-methyl-2*H*-chromen-7-yloxy) acetic acid **3** (Abd El-Fattah *et al.,* 2011) which was esterified using a series of alcohols in the presence of iodine (Ramalinga *et al.,* 2002; Jereb *et al.,* 2009) to yield a new series of coumarin esters **4a–j** (Scheme 1; Table 1).

In the ^{1}H-NMR spectrum of compound **2**, a 3H triplet was observed at δ_H 1.25 ppm ($J = 7.2$ Hz) due to the ester CH_3 protons and a quartet at δ_H 4.23 ppm ($J = 7.2$ Hz) due to the ester CH_2 protons. The isolated CH_2 protons were observed downfield as a singlet (2H) at δ_H 4.63 ppm. The ^{13}C-NMR spectrum analysis for compound **2**, combined with the information from ^{1}H-NMR experiment, can be considered enough to guide future synthetic work. The ^{13}C NMR spectrum of this compound confirms the proposal structure by the observation of the signals at δ_C 13.6 (C-4′), 61.1 (C-3′) and 167.4 (C-2′) ppm. The ^{13}C-NMR spectrum analysis for compound **2**, combined with the information from ^{1}H-NMR experiments, can be considered enough to guide future synthetic work.

In the ^{1}H-NMR spectrum of compound **3**, the CH_2 protons were observed a singlet (2H) at δ_H 4.78 ppm and the CH_3 of the coumarin moiety appeared at δ_H 2.39 ppm. On the same spectrum, we noticed the appearance of a new signal at δ_H 13.07 ppm corresponding to the OH group of the acetic acid fragment. The ^{13}C-NMR spectrum of compound **3** shows in particular a signal at δ_C 169.6 ppm attributable to the carbonyl of the acid group (C-2′).

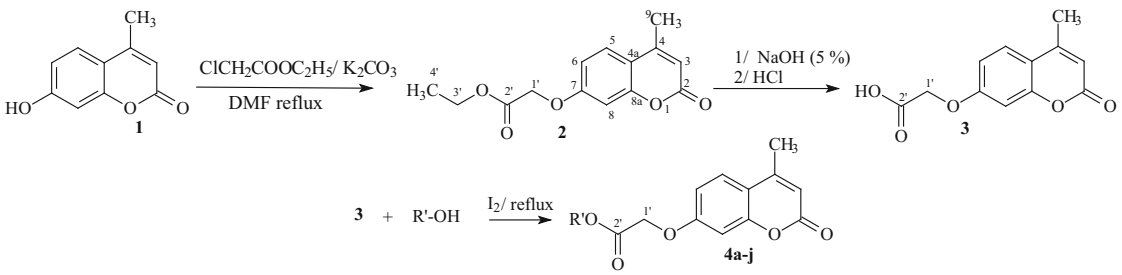
The structure of these compounds (**4a–j)** was established on the basis of their analytical data. Thus, mass spectrum of **4a** (as an example) gave a pseudo-molecular ion peak $[M + H]^+$ at *m/z* 305.1381, which is consistent with the molecular formula ($C_{17}H_{21}O_5$). In addition to the signals corresponding to the protons introduced by 4-methylumbelliferone, new signals related to the alcohol used were observed in the ^{1}H-NMR spectrum. Examination at 300 MHz showed a multiplet at δ_H 3.99 (2H, H-5′) and also a multiplet at δ_H 0.87 (6H, H-6′, H-7′). The ^{13}C-NMR spectrum confirmed the above spectral data by the observation of new signals at δ_C 11.2, 16.3, 25.9, 34.1 and 70.1 ppm relative to carbons of the alcohol used. The same spectrum showed a signal at δ_C 168.1 ppm attributable to the carbonyl of the ester group (C-2′).

In the second step, we have subjected the key intermediate **2** to reaction with an aqueous solution of hydrazine in ethanol at reflux (Al-Amiery *et al.,* 2011b). The so-formed hydrazide **5**, which belongs to a class of intermediates, known to be highly reactive and used as precursors for the synthesis of new nitrogen's compounds (Abdel-Aziz *et al.,* 2007) was further converted into coumarin derivatives **6** and **7** by treatment with a series of aromatic aldehydes (Scheme 2; Table 2) and cyclic anhydrides, respectively (Scheme 3; Table 3).

The ^{1}H-NMR spectrum of the intermediate **5** showed characteristic signals at δ_H 5.02, 4.38 and 9.45 ppm corresponding to the methylene (C-1′), NH_2 and NH groups, respectively. Its ^{13}C-NMR spectrum reinforced this structure by the appearance of the signal of (C-2′) at δ_C 169.6 ppm and the disappearance of the two signals at δ_C 13.6 (C-4′) and 61.1 (C-3′) ppm attributed to the ethoxy carbons in compound **2**.

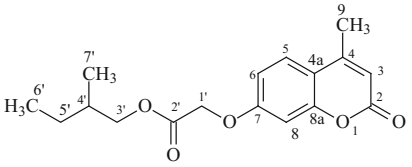
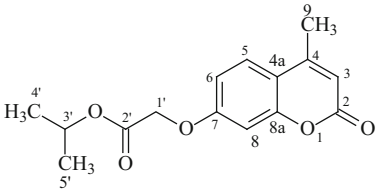
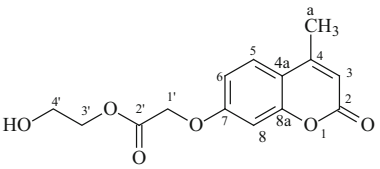
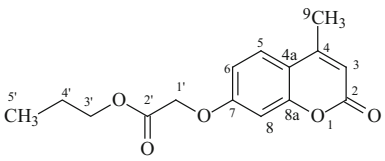
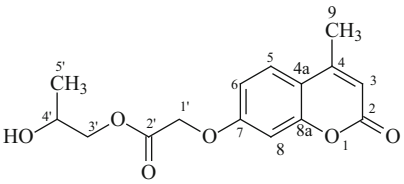
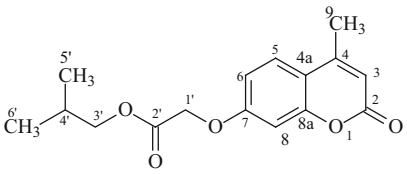
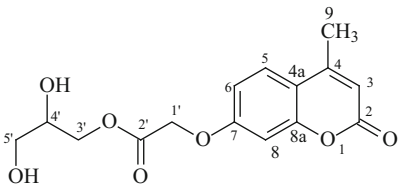
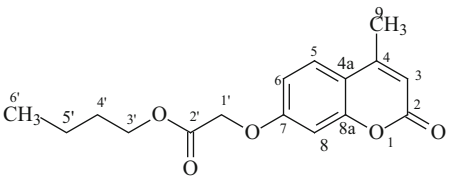
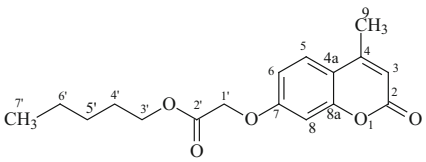
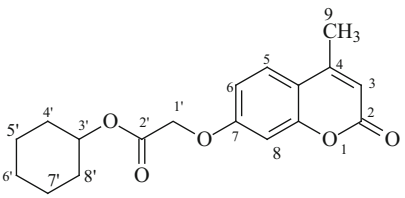
Compound **6b** was obtained as a white powder. Its ESHRMS gave pseudo-molecular ion peak $[M + H]^+$ at *m/z* 327.0975, which is consistent with the molecular formula $C_{17}H_{14}N_2O_5$. Thus, the ^{1}H-NMR spectrum of **6b** showed the absence of the NH_2 protons at δ_H 4.38 ppm, and the appearance of a new characteristic singlet at δ_H 7.83 ppm, assignable to the proton –N = CH and the presence of the NHCO proton resonating at δ_H 11.63 ppm.

Also the structure of compound **7** has been assigned from their analytical data. In fact the ESHRMS of



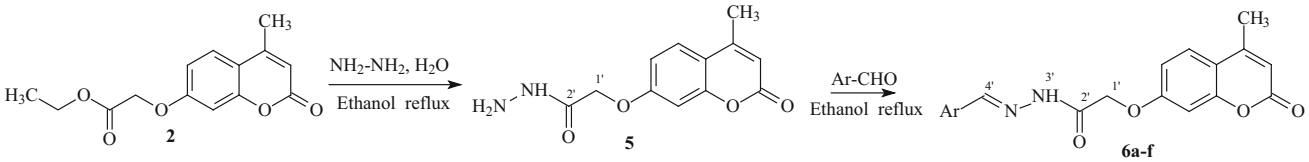
Scheme 1 Synthesis of new coumarin esters **4a–j**

Table 1 Synthesis of compounds **4a–j**

Entry	Product	Entry	Product
1	 **4a**	**6**	 **4f**
2	 **4b**	**7**	 **4g**
3	 **4c**	**8**	 **4h**
4	 **4d**	**9**	 **4i**
5	 **4e**	**10**	 **4j**

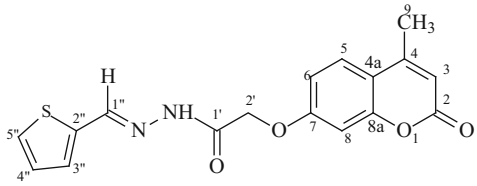
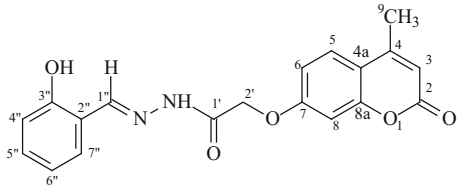
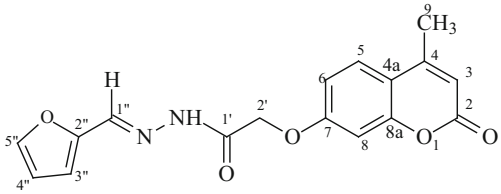
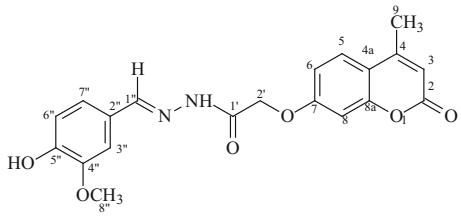
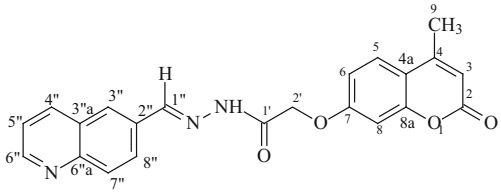
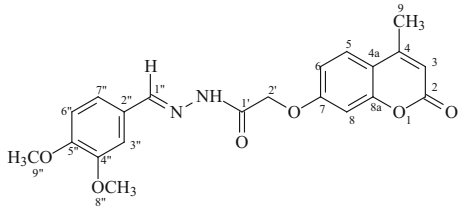
Conditions: acid (0.1g, 0.4 mmol), alcohol (10 mL), iodine (5 mg), reflux 2-24 h

compound **7a** gave pseudo-molecular ion peak $[M + H]^+$ at *m/z* 377.0777, which is consistent with the molecular formula $C_{21}H_{19}O_6N_2$. Furthermore, the ^{1}H NMR spectrum of this compound was compatible with the proposed structure. In addition to the signals corresponding to most of the protons introduced by the hydrazide **5**, we note the presence of a new multiplet (4H) at δ_H 8.01 ppm relative to the aromatic protons of the phthalic anhydride. The ^{13}C-NMR spectrum confirmed the above spectral data by the observation of new signals at δ_C 129.3, 135.4, 154.5 and



Scheme 2 Synthesis of hydrazone **6a–f**

Table 2 Synthesis of compounds **6a–f**

Entry	Product	Entry	Product
1	 **6a**	4	 **6d**
2	 **6b**	5	 **6e**
3	 **6c**	6	 **6f**

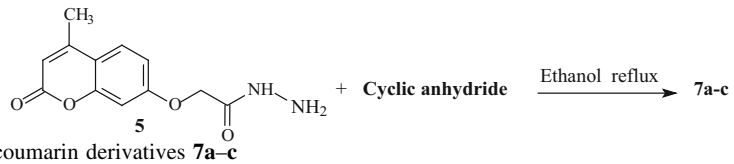
Conditions: hydrazide **5** (4 mmol), aromatic aldehydes (1eq), ethanol (20 mL), 2h, reflux

164.9 ppm relative to carbons of the phthalic anhydride moiety.

Biological activity

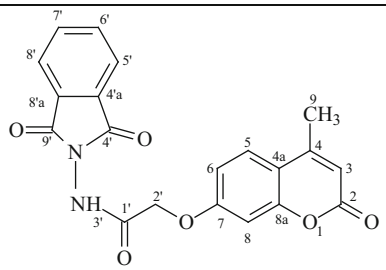
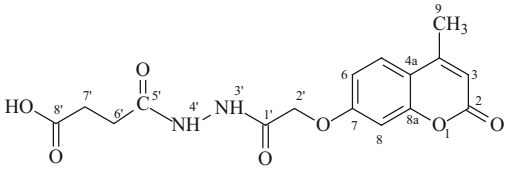
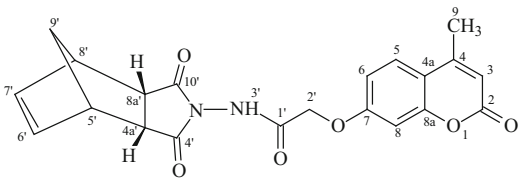
Antimicrobial activity

The serious medical problem of bacterial and fungal resistance and the rapid rate of its development has led to increasing levels of resistance to classical antibiotics (Rice *et al.,* 1999; Al-Amiery *et al.,* 2009; Kadhum *et al.,* 2011), and the discovery and development of effective antibacterial and antifungal drugs with novel mechanisms of action have become urgent tasks for infectious disease research programs (Kumar *et al.,* 2011b). All the synthesized compounds were analyzed on what concerns their antimicrobial inhibition (Table 4). The antimicrobial inhibition was determined using the disc diffusion method



Scheme 3 Synthesis of novel coumarin derivatives **7a–c**

Table 3 Synthesis of compounds **7a–c**

Entry	Product	Entry	Product
1	 **7a**	3	 **7c**
2	 **7b**		

Conditions: hydrazide **5** (4 mmol), cyclic anhydre (1eq), ethanol (20 mL), 8h, reflux

described in the literature (Marmonier, 1987; Barry and Thornsberry, 1991).

According to the results given in Table 4, most compounds have displayed good antifungal activity against *Botrytis cinerea* and *Fusarium oxysporum* f. sp. *lycopersici*. Compounds **4a** and **4g** exhibited an important inhibitory activity against *B. cinerea* (IZ = 36 and 39 mm, respectively), while compound **4j** was found to be the most active against *F. oxysporum* f. sp. *lycopersici* (IZ = 16 mm). The compounds **4a**, **4i** and **7a** showed moderate activity against *Aspergilus niger* (IZ = 6–8 mm).

From the data of the antibacterial activity, few compounds (**4f**, **4h**, **4i**, **6e**, **7a** and **7c**) were found to be active against *Pseudomonas* sp. (Pa 499) and *Bacillus* sp (Bp 420) (IZ = 5–8 mm) (Table 4). However, compound **7b** exhibited an interesting antibacterial activity against *Pseudomonas* sp. (IZ = 12 mm) which was comparable to that obtained with the standard reference (ampicillin) used.

Some results prove consistent with several studies showing that coumarins and some of their derivatives exhibited an important antimicrobial activity (Lin *et al.,* 2012; Singh *et al.,* 2010).

Moreover, it was indicated that the combination of the coumarin skeleton with some nitrogen-containing heterocyclic moieties could significantly increase their antimicrobial efficiency (Keri *et al.,* 2009; Ronad *et al.,* 2010).

The antimicrobial activity of the compounds **4a–j** depends on the structure of the fragment introduced by the alcohol. Generally, the esterification does not seem beneficial for such antibacterial activity. Secondly, the behavior of the fungi used, such as *B. cinerea*, to compounds **4a–j** appears complex, but in all cases the hydroxyl group or groups remained free in certain alcohols used (cases of **4b**, **4c** and **4d**) did not appear an interesting factor that brings activity. The relatively high activity of compound **6d** against *F. oxysporum* (IZ = 15 mm) could be explained by the presence of the free phenol function. The ortho-position of the latter allows the formation of a possible hydrogen bond with the free electron pair of the sp2 hybridized nitrogen atom of the hydrazone function, thus

Table 4 Antimicrobial activity of coumarin derivatives **4a–j, 6a–f** and **7a–c**

Bacteria			Fungi		
Compound	*Pseudomonas* sp. (Pa 499)	*Bacillus* sp. (Bp 420)	*Aspergillus niger*	*Botrytis cinerea*	*Fusarium oxysporum* f. sp. *lycopersici*
Inhibition zone (IZ) in mm					
4a	–	–	7	36	–
4b	–	–	–	–	12
4c	–	–	–	–	11
4d	–	–	–	11	13
4e	–	–	–	11	–
4f	5	–	–	–	12
4g	–	–	–	39	11
4h	5	–	–	–	12
4i	–	6	8	–	12
4j	–	–	–	–	16
6a	–	–	–	10	–
6b	–	–	–	–	11
6c	–	–	–	–	11
6d	–	–	–	13	15
6e	8	–	–	–	11
6f	–	–	–	–	–
7a	6	–	6	12	11
7b	12	6	–	15	13
7c	5	–	–	–	12
Ampicillin	10	10	–	–	–
Carbendazim	–	–	10	30	10

– No inhibition zone observed

further stabilizing the molecule; this stability may be at the origin of the activity of this derivative. On the other hand, the para-position of this phenol function to the hydrazone in compound **6e** and the presence of a methoxyl group certainly explain the attenuation of this activity against the same strain (IZ = 11 mm). The relatively high activity of compound **7b** toward bacteria and fungi used except *A. niger* compared to its analogue **7a** could be partly explained by the non-aromatic bicyclic structure of the moiety introduced by the anhydride used. The open structure of the fragment introduced by the succinic anhydride used in the preparation of compound **7c** and the emergence of a carboxylic acid function were without significant effect on its antimicrobial activity.

Conclusion

In conclusion, the present study provides a novel and efficient synthetic route to the preparation of new coumarin esters, hydrazone and novel coumarin derivatives from 4-methylumbelliferone. The results showed that some of the synthetic compounds possessed good antibacterial and antifungal activities. Most of the compounds might be promising for any investigations to develop new effective antibacterial and antifungal drugs.

Experimental section

Chemistry

Melting points were determined on a Büchi 510 apparatus using capillary tubes. Mass spectra were obtained with ESI-TOF (LCT Premier XE, Waters) using the reflectron mode in the positive ion mode. Leucine-enkephalin peptide was employed as the LockSpray lockmass. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Bruker AM-300 spectrometer, using $CDCl_3$ and DMSO-d_6 as solvents and non-deuterated residual solvents as internal standards. Chemicals shifts (δ) are given in parts per million (ppm) and coupling constants (*J*) in Hertz. Commercial TLC plates (Silica gel 60, F254, SDS) were used to monitor the progress of the reaction. Column chromatography was

performed with silica gel 60 (particle size 40–63 μm, SDS). The starting materials **2**, **3** and **5** were prepared according to the literature (Ramesh *et al.,* 2008; Al-Amiery *et al.,* 2011).

*Preparation of compounds **4a–j***

A mixture of acid **3** (0.1 g, 0.4 mmol), alcohol (10 ml) and iodine (5 mg,) was boiled under reflux for 2 to 24 h. The progress of the reaction was monitored by TLC. The solvent (excess of alcohol) was evaporated in vacuo. The mixture was extracted twice with the appropriate solvent and then dried over Na_2SO_4. After removing the solvent under reduced pressure, the residue thus obtained was purified by column chromatography on silica gel eluted with a mixture PE/EtOAc (8:2) to give the desired carboxylic esters **4a–j**.

*Ethyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetate (**2**)*

Yellow solid, yield 72 %, mp 102 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 1.25 (t, 3H, J = 7,2 Hz, CH_3), 2.33 (s, 3H, CH_3), 4.23 (q, 2H, J = 7,2 Hz, H-3′), 4.63 (s, 2H, H-1′), 6.06 (s, 1H, H-3), 6.69 (d, 1H, J = 2,7 Hz, H-8), 6.83 (dd, 1H, J_1 = 8,7 Hz, J_2 = 2,7 Hz, H-6), 7.46 (d, 1H, J = 8,7 Hz, H-5). ^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 13.6 (CH_3-4′), 18.0 (CH_3-9), 61.1 (CH_2-3′), 64.8 (CH_2-1′), 101.2 (CH-8), 111.8 (CH-6 + C-3), 113.8 (C-4a), 125.3 (CH-5), 151.9 (C-8a), 154.4 (C-4), 160.1 (C-7 + C-2), 167.4 (C-2′).

*2-(4-Methyl-2-oxo-2H-chromen-7-yloxy) acetic acid (**3**)*

White solid, yield 67 %, mp 206 °C (EtOH).^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 2.39 (s, 3H, CH_3), 4.78 (s, 2H, H-2′), 6.21 (s, 1H, H-3), 6.98 (dd, 1H, J_1 = 8,4 Hz, J_2 = 2,4 Hz, H-8), 7.69 (d, 1H, J = 8,7 Hz, H-5), 13.07 (s, 1H, OH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_c 18.1 (CH_3-9), 64.8 (CH_2-1′), 101.5 (CH-8), 111.4 (CH-6), 112.2 (CH-3), 113.5 (C-4a), 126.5 (CH-5), 153.3 (C-8a), 154.5 (C-4), 160.7 (C-7 + C-2), 169.6 (C-2′).

*2-Methylbutyl 2-(4-methyl-2-oxo-2H-chromen-7- yloxy) acetate (**4a**)*

White solid, yield 53 %, mp 301 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 2.37 (s, 3H, CH_3), 0.87 (m, 6H, H-6′ + H-7′), 1.13 (m, 1H, H-3′a), 1.35 (m, 1H, H-3′b), 1.70 (m, 1H, H-4′), 3.99 (m, 2H, H-5′), 4.69 (s, 2H, H-1′), 6.12 (s, 1H, H-3), 6.75 (d, 1H, J = 2.4 Hz, H-8), 6.83 (dd, 1H, J_1 = 8.7 Hz, J_2 = 2.7 Hz, H-6), 7.46 (d, 1H, J = 8.7 Hz, H-5).

^{13}C-NMR (75 MHz $CDCl_3$): δ_c 11.2 (CH_3-6′), 16.3 (CH_3-7′), 18.7 (CH_3-9), 25.9 (CH_2-5′), 34.1 (CH-4′), 65.2 (CH_2-1′), 70.1 (CH_2-3′), 101.7 (CH-8), 112.4 (CH-6), 114.3 (CH-3), 125.8 (CH-5), 152.5 (C-8a), 155.0 (C-4), 160.5 (C-7), 161.1 (C-2), 168.2 (C-2′). ESI-HRMS *m/z* $[M + H]^+$ calcd. for $(C_{17}H_{21}O_5)^+$:305.1389 found: 305.1381.

*2-Hydroxyethyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetate (**4b**)*

White solid, yield 70 %, mp 308 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 2.33 (s, 3H, CH_3), 3.82 (t, 2H, J_1 = 3.3 Hz, J_2 = 1.2 Hz, H-4′), 4.30 (t, 2H, J_1 = 3.3 Hz, J_2 = 1.3 Hz, H-3′), 4.68 (s, 2H, H-1′), 6.10 (s, 1H, H-3), 6.72 (d, 1H, J = 2.4 Hz, H-8), 6.87 (dd, 1H, J_1 = 8.7 Hz, J_2 = 2.4 Hz, H-6), 7.45 (d, 1H, J = 8.7 Hz, H-5). ^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 18.7 (CH_3-9), 60.8 (CH_2-4′), 65.2 (CH_2-1′), 66.9 (CH_2-3′), 101.7 (CH-8), 112.6 (CH-6), 114.5 (CH-3); 125.9 (CH-5), 152.5 (C-8a), 155.1 (C-4), 160.5 (C-7), 161.2 (C-2), 168.3 (C-2′). ESI-HRMS $[M + H]^+$ calcd. for $(C_{14}H_{15}O_6)^+$: 279.0790 found: 279.0796.

*2-Hydroxypropyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetate (**4c**)*

White solid, yield 72 %, mp 219 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 1.22 (m, 3H, H-5′), 2.38 (s, 3H, CH_3), 3.64 (m, 1H, H-4′), 4.12 (m, 2H, H-3′), 4.76 (s, 2H, H-1′), 6.14 (1H, s, H-3), 6.79 (d, 1H, J = 2.4 Hz, H-8), 6.87 (dd, 1H, J_1 = 8.7 Hz, J_2 = 2.4 Hz, H-6), 7.50 (d, 1H, J = 8.7 Hz, H-5). ^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 18.2 (CH_3-9), 18.7 (CH_3-5′), 64.7 (CH-4′), 69.3 (CH_2-1′), 73.0 (CH_2-3′), 101.3 (CH-8), 112.1 (CH-6), 114.0 (CH-3), 125.3 (CH-5), 151.9 (C-8a), 154.5 (C-4), 160.5 (C-7), 167.5 (C-2), 167.6 (C-2′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{15}H_{16}O_6)^+$: 292.0947 found: 292.0950.

*2,3-Dihydroxypropyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetate (**4d**)*

White solid, yield 22 %, mp 208 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 2.40 (s, 3H, CH_3), 3.70 (dd, 2H, J_1 = 15.2 Hz, J_2 = 6 Hz, H-5′), 4.15 (dd, 2H, J = 2.1 Hz, J = 11.4 Hz, H-3′), 4.08 (m, 1H, H-4′), 4.78 (s, 2H, H-1′), 6.18 (s, 1H, H-3), 6.82 (d, 1H, J = 2.4 Hz,, H-8), 6.95 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz,, H-6), 7.55 (d, 1H, J = 8.7 Hz,, H-5) ^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 20.2 (CH_3-9), 62.4 (CH_2-5′), 64.1 (CH-4′), 66.1 (CH_2-3′), 70.1 (CH_2-1′), 102.0 (CH-8), 110.9 (CH-6), 112.2 (CH-3), 115.3 (C-4a), 128.8 (CH-5), 152.0 (C-8a), 155.1 (C-4), 160.7 (C-7) 162.4 (C-2), 168.9 (C-2′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{15}H_{17}O_7)^+$: 309.0896 found: 309.0899.

*Pentyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetate **(4e)***

White solid, yield 72 %, mp 202 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 1.18 (m, 8H, H-3′ + H-4′ + H-5′ + H-6′), 1.59 (m, 3H, CH_3), 2.31 (s, 3H, CH_3), 4.61 (s, 2H, H-1′), 6,05 (s, 1H, H-3), 6.69 (d, 1H, $J = 2.4$ Hz, H-8), 6.82 (dd, 1H, $J_1 = 8.7$ Hz, $J_2 = 2.7$ Hz, H-6), 7.44 (d, 1H, $J = 8.7$ Hz, H-5). ^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 18.7 (CH_3-9), 18.6 (CH_3-7′), 22.2 (CH_2-6′), 29.4 (CH_2-5′), 29.6 (CH_2-4′), 65.7 (CH_2-1′), 67.7 (CH_2-3′), 101.7 (CH-8), 104.9 (CH-6), 114.3 (C-3), 125.7 (CH-5), 152.4 (C-8a), 155.1 (C-4), 160.6 (C-2), 160.9 (C-7), 168.1 (C-2′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{17}H_{21}O_5)^+$: 305.1311 found: 305.1318.

*Isopropyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetate **(4f)***

White solid, yield 78 %, mp 218 °C (EtOH).^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 1.20 (d, 6H, $J = 6$ Hz, H-4′ + H-5′), 2.31 (s, 3H, CH_3), 4.55 (s, 2H, H-1′), 5.07 (hept, 1H, $J = 6$ Hz, H-3′), 6.07 (s, 1H, H-3), 6.71 (d, 1H, $J = 2.7$ Hz, H-8), 6.83 (dd, 1H, $J_1 = 8.4$ Hz, $J_2 = 2.4$ Hz, H-6), 7.42 (d, 1H, $J = 8.4$ Hz, H-5). ^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 21.2 (CH_3-4′ + 5′), 18.1 (CH_3-9), 65.1 (CH_2-1′), 69.2 (CH-3′), 101.2 (CH), 112.0 (CH-6 + C-3), 113.8 (C-4a), 125.2 (CH-5), 151.8 (C-8a), 154.6 (C-4), 160.2 (C-2), 160.5 (C-7), 177.0 (C-2′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{15}H_{17}O_5)^+$: 277.0998 found: 277.0993.

*Propyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetate **(4 g)***

White solid, yield 71 %, mp 212 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 0.91 (t, 3H, $J = 7.5$ Hz, CH_3), 1.65 (m, 2H, H-4′), 2.36 (s, 3H, CH_3), 4.15 (t, 2H, $J = 6.6$ Hz, H-3′), 4.67 (s, 2H, H-1′), 6.10 (s, 1H, H-3), 6.73 (d, 1H, $J = 2.7$ Hz, H-8), 6.88 (dd, 1H, $J_1 = 8.4$ Hz, $J_2 = 2.4$ Hz, H-6), 7.47 (d, 1H, $J = 8.4$ Hz, H-5).^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 21.8 (CH_3-9), 18.6 (CH_3-5′), 10.2 (CH_2-4′), 65.2 (CH_2-3′), 67.2 (CH_2-1′), 101.7 (CH-8), 112.4 (CH-6), 114.3 (C-3), 125.2 (CH-5), 152.5 (C-8a), 154.9 (C-4), 160.6 (C-7), 161.1 (C-2), 168.1 (C-2′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{15}H_{17}O_5)^+$: 277.1076 found: 277.1073.

*Isobutyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetate **(4h)***

White solid, yield 79 %, mp 228 °C (EtOH).^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 0.85 (d, 6H, $J = 3.9$ Hz, H-5′ + H-6′), 2.32 (s, 3H, CH_3), 1.89 (hept, 1H, $J = 6.9$ Hz, H-4′), 3.92 (d, 2H, $J = 6.6$ Hz, H-3′), 4.63 (s, 2H, H-1′), 6.07 (s, 1H, H-3), 6.71 (d, 1H, $J = 2.7$ Hz, H-8), 6.83 (dd, 1H, $J_1 = 8.4$ Hz, $J_2 = 2.4$ Hz, H-6), 7.43 (d,1H, $J = 8.4$ Hz, H-5). ^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 27.7 (CH_3-5′ + 6′), 18.6 (CH_3-9), 65.3 (CH_2-3′), 71.6 (CH_2-1′), 101.7 (CH-8), 112,5 (CH-6), 114.4 (CH-3), 125.7 (CH-5), 152.3 (C-8a), 155.1 (C-4), 160.7 (C-7), 161.0 (C-2), 168.1 (C-2′).ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{16}H_{19}O_5)^+$: 291.1154 found: 291.1151.

*Butyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetate **(4i)***

White solid, yield 65 %, mp 221 °C (EtOH). ^{1}H-NMR (300 MHz $CDCl_3$, 300 MHz): δ_H 0.85 (t, 3H, $J_1 = 7.5$ Hz, $J_2 = 7.2$ Hz, CH_3), 2.40 (s, 3H, CH_3), 1.33 (m, 2H, H-5′), 1.65 (m, 2H, H-4′) 4.22 (t, 2H, $J_1 = 6.9$ Hz, $J_2 = 6.6$ Hz, H-3′), 4.69 (s, 2H, H-1′), 6.15 (s, 1H, H-3), 6.78 (d, 1H, $J_1 = 2.7$ Hz, H-8), 6.92 (dd, 1H, $J_1 = 8.4$ Hz, $J_2 = 2.4$ Hz, H-6), 7.51 (d, 1H, $J = 8.4$ Hz, H-5).^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 13.6 (CH_3-6′), 18.7 (CH_2-5′), 19.0 (CH_3-9), 29.4 (CH_2-4′), 65.3 (CH_2-3′), 65.5 (CH_2-1′), 101.7 (CH-8), 112.5 (CH-6), 114.4 (C-3), 125.8 (CH-5), 152.4 (C-8a), 155.1 (C-4), 160.6 (C-7), 161.1 (C-2), 168.1 (C-2′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{16}H_{19}O_5)^+$: 291.1154 found: 291.1159.

*Cyclohexyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetate **(4j)***

White solid, yield 69 %, mp 210 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 1.33 (m, 2H, H-6′), 1.42 (m, 2H, H-5′), 1.65 (m, 2H, H-4′), 1.89 (m, 2H, H-8′), 2.40 (s, 3H, CH_3), 4.65 (s, 2H, H-1′), 4.90 (m, 1H, H-3′), 6.15 (s, 1H, H-3), 6.78 (d, 1H, $J = 2.7$ Hz, H-8), 6.93 (dd, 1H, $J_1 = 8.7$ Hz, $J_2 = 2.7$ Hz, H-6), 7.55 (d, 1H, $J = 8.7$ Hz, H-5). ^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 19.2 (CH_3-9), 22.4 (CH_2-8′), 23,4 (CH_2-6′), 25.3 (CH_2-5′), 31.0 (CH_2-4′), 67.8 (CH_2-1′), 72,2 (CH_2-7′), 78.9 (CH-3′), 103.2 (CH-8), 112.0 (CH-6), 112.1 (C-4a), 116.5 (CH-3), 128.7 (CH-5), 155.3 (C-8a), 158.0 (C-4), 160.3 (C-7), 161.8 (C-2), 169.2 (C-2′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{18}H_{21}O_5)^+$: 316.1311 found: 316.1316.

*2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetohydrazide **(5)***

White solid, yield 63 %, mp 212 °C (EtOH). ^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 2.39 (s, 3H, CH_3), 4.38 (s, 2H, NH_2), 5.02 (s, 2H, H-1′), 6.23 (s, 1H, H-3), 7.0 (m, 2H, H-6 + H-8), 7.68 (d, 1H, H-5), 9.45 (s, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_c 18.1 (CH_3-9), 64.9 (CH_2-1′), 102.0 (CH-8), 111.6 (CH-6), 112.3 (CH-3), 113.5 (C-4a), 126.4 (CH-5), 153.5 (C-8a), 154.5 (C-4), 160.8 (C-7 + C-2), 169.9 (C-2′).

Preparation of compounds 6a–f: A mixture of compound ***5***

(4 mmol) and the appropriate aromatic aldehyde (1 eq) was refluxed in ethanol (20 mL) for 2 h. The excess of solvent was then removed under reduced pressure, the precipitate formed after cooling was collected by filtration and recrystallized from ethanol to give compounds **6a–f**.

*(E)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)-N'-(thiophen-2-ylmethylene)acetohydrazide (**6a**)*

White solid, yield 66 %, mp 268 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 2.33 (s, 3H, CH_3), 4.86 (s, 2H, H-2′), 6.22 (s, 1H, H-3), 6.65 (s, 1H, H-4′'), 6,94-7,09 (m, 3H, H-3″ + H-6 + H-8), 7.78 (d, 1H, J = 8,7 Hz, H-5), 7.92 (s, 1H, H-1″), 7.98 (s, 1H, H-5″), 11.63 (s, 1H, NH). ^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 18.1 (CH_3-9), 66.7 (CH_2-2′), 101.6 (CH-8), 113.4 (CH-3 + C-6), 113.7 (C-4a), 125.0 (CH-1″), 125.8 (CH-5″), 127.2 (CH-4″), 128.0 (CH-3″),, 145.4 (C-8a), 153.3 (C-4), 144.5 (C-2″), 160.6(C-7 + 2), 168.8 (C-1′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{17}H_{15}N_2O_4S)^+$: 342.0674, found: 342.0677.

*(E)-N'-(furan-2-ylmethylene)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetohydrazide (**6b**)*

White solid, yield 76 %, mp 248 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 2.39 (s, 3H, CH_3), 4.80 (s, 2H, H-2′), 6.23 (s, 1H, H-3), 6.62 (s, 1H, H-4″), 6,92-7,06 (m, 3H, H-3″ + H-6 + H-8), 7.71 (d, 1H, J = 8,7 Hz, H-5), 7.83 (s, 1H, H-1″), 7.91 (s, 1H, H-5″), 11.63 (s, 1H, NH). ^{13}C-NMR (75 MHz, $CDCl_3$): δ_c 18.1 (CH_3-9), 65.8 (CH_2-2′), 101.8 (CH-8), 113.5 (CH-3 + C-6), 114.2 (C-4a), 125.4 (CH-1″), 125.8 (CH-5″), 127.2 (CH-4″), 128.0 (CH-3″),, 145.4 (C-8a), 153.3 (C-4), 144.5 (C-2″), 160.6(C-7 + 2), 168.8 (C-1′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{17}H_{15}N_2O_5)^+$: 327.0981, found: 327.0975.

*(E)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)-N'-(quinolin-6-ylmethylene)acetohydrazide (**6c**)*

White solid, yield 84 %, mp 238 °C (EtOH). ^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 2.40 (s, 3H, CH_3), 5.41 (s, 2H, H-2′), 6.21 (s, 1H, H-3), 7.0 (m, 2H, H-6 + H-8), 7.69-9.0 (m, 8H, H-5 + H-1″ + H-3″ + H-4″ + H-5″ + H-6″ + H-7″ + H-8″), 12.03 (s, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_c 18.1 (CH_3-9), 66.7 (CH_2-2′), 101.6 (CH-8), 113.4 (CH-3 + C-6), 113.7 (C-4a), 119.7 (CH-8″), 120.2 (C-2″), 123.9 (CH-5), 124.5 (CH-5″), 126.4-126.5 (CH-7″ +3″a), 126.6 (CH-5″), 145.4 (C-8a), 153.3 (C-4), 154.5 (CH-1″), 160.6 (C-7 + 2), 164.2 (CH-6″), 168.8 (C-1′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{22}H_{18}O_4N_3)^+$: 388.1297 found: 388.1301.

*(E)-N'-(2-hydroxybenzylidene)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetohydrazide (**6d**)*

White solid, yield 82 %, mp 212 °C (EtOH). ^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 2.19 (s, 3H, CH_3), 5.28 (s, 2H, H-2′), 6.21 (s, 1H, H-3), 7.0 (m, 2H, H-6 + H-8), 7.05-8.22 (m, 5H, H-1″ + H-4″ + H-5″ + H-6″ +H-7″), 11.01 (s, 1H, OH), 11.85 (s, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_c 18.1 (CH_3-9), 64.8 (CH_2-2′), 101.5 (CH-8), 111,4 (CH-6), 112.2 (CH-3), 113.5 (C-4a), 120.2 (C-2″); 121.5 (CH-4″), 123.0 (CH-6″), 127.2 (CH-7″), 130.1 (CH-5″), 126.5 (CH-5), 153.3 (C-8a), 154.5 (C-4), 156.0 (CH-1″), 160,1 (C-3″), 163,5 (C-2 + 7), 168,1 (C-1′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{19}H_{17}O_5N_2)^+$: 353.1059 found: 353.1063.

*(E)-N'-(4-hydroxy-3-methoxybenzylidene)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetohydrazide (**6e**)*

White solid, yield 78 %, mp 229 °C (EtOH). ^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 2.19 (s, 3H, CH_3), 3.85 (s, 3H, H-8″), 5.28 (s, 2H, H-2′), 6.21 (s, 1H, H-3), 7.02 (m, 2H, H-6 + H-8), 7.11-7.22 (m, 4H, H-1″ + H-3″ + H-5″ + H-7″), 11.01 (s, 1H, OH), 11.85 (s, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_c 18.1 (CH_3-9), 56.2 (OCH_3-8″), 66.5 (CH_2-2′), 113.0 (C-5″), 126.5 (CH-5), 130.1 (CH), 131.4 (CH), 153,3 (C-8a), 154.5 (C-4), 156,1 (CH-1″); 160,6 (C-7); 160,1 (CH-7″); 160,0 (CH-3″); 163.5 (C-2 + 7), 168.1 (C-1′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{20}H_{19}O_6N_2)^+$: 383.1165 found: 383.1169.

*(E)-N′-(3,4-dimethoxybenzylidene)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetohydrazide (**6f**)*

White solid, yield 81 mp 222 °C (EtOH). ^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 2.19 (s, 3H, CH_3), 3.85 (s, 3H, H-8″), 3.91 (s, 3H, H-9″), 5.28 (s, 2H, H-2′), 6.21 (d, 1H, J = 0.9 Hz, H-3), 7.02 (m, 2H, H-6 + H-8), 7.10-7.24 (m, 4H, H-1″ + H-3″ + H-6″ + H-7″), 11.85 (s, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_c 18.1 (CH_3-9), 65.2 (OCH_3-8″), 66.5 (CH_2-2′), 163.5 (C-2), 160.8 (C-7), 160.0 (CH-1″), 131.4 (C-2″), 113.0 (CH-3″), 155.0 (C-4a), 152.0 (C-4″), 148.0 (C-5″), 130.1 (CH-6″), 121.0 (CH-7″), 126.5 (CH-5), 152.3 (C-8a), 154.5 (C-4), 156.0 (CH-1″), 168.5 (C-1′).ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{21}H_{23}O_6N_2)^+$: 397.1321 found: 397.1325.

*Preparation of compounds **7a–c***

A mixture of hydrazide **5** (100 mg, 4 mmol), cyclic anhydride (1 eq) and 20 mL of ethanol was refluxed. The progress of the reaction was monitored by TLC. Once the reaction is completed, the precipitate was filtered and recrystallized from ethanol to obtain the desired products, **7a–c**.

*N-(1,3-dioxoisoindolin-2-yl)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetamide (**7a**)*

White solid, yield 26 %, mp 280 °C (EtOH). ^{1}H-NMR (DMSO-d_6, 300 MHz): δ_H 2.42 (s, 3H, CH_3), 5.0 (s, 2H, H-2′), 6.27 (s, 1H, H-3), 7.10 (m, 2H, H-6 + H-8), 7.76 (d, 1H, J = 8.4 Hz, H-5), 8.01 (m, 4H, H-5′ + H-6′ + H-7′ + H-8′), 11.10 (s, 1H, NH).

^{13}C-NMR (75 MHz, DMSO-d_6): δ_c 18.2 (CH_3-9), 66.1 (CH_2-2′), 111.6 (CH-8), 112.6 (CH-3 + C-6), 115.0 (C-4a), 123.9 (CH-5), 129.3 (CH-5′ + C-8′), 135.4 (CH-6′ + C-7′), 160.1-160.3 (C-2 + C-7), 166.9 (C-1′), 101.7 (CH-8), 153.4 (C-8a), 154.5 (C-4′a + C-9′a), 164.9 (C-9′ + C-4′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. For $(C_{20}H_{13}O_6N_2)^+$: 377.0787 found: 377.0777.

*N′-(2,9-dioxo ([2,2.1] hept-5-en)-2-yl)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetohydrazide (**7b**)*

White solid, yield 66 %, mp 270 °C (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 1.71 (m, 2H, H-9′), 2.41 (s, 3H, CH_3), 3.41 (m, 2H, H-4′a + H-8′a), 3.44 (m, 2H, H-8′ + H-5′), 4.81 (s, 2H, H-2′), 6.18 (s, 1H, H-3), 6.22 (s, 1H, H-6′), 6.31 (s, 1H, H-7′), 7.02 (m, 2H, H-6 + H-8), 7.80 (d, 1H, J = 8.7 Hz, H-5), 8.53 (s, 1H, NH). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_c 18.7 (CH_3-9), 22.1 (CH_2-9′), 44.4 (C-4′a + C-8′a), 45.1 (CH-8′ + C-5′), 67.1 (CH_2-2′), 111.6 (CH-8), 112.6 (CH-3 + C-6), 155.0 (C-4a), 123.9 (CH-5), 134.8 (CH-6′ + C-7′), 152.5 (C-8a), 160.1–160.6 (C-2 + C-7), 160,9 (C-4′), 161.1 (C-10′), 173.6 (C-1′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{21}H_{19}O_6N_2)^+$: 395.1243 found: 395.1240.

*4-(2-(2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetyl)hydrazinyl)-4-oxobutanoic acid (**7c**)*

White solid, yield 76 %, mp 266 °C (EtOH). ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 2.41 (s, 3H, CH_3), 2.54 (m, 4H, H-6′ + H-7′), 4.81 (s, 2H, H-2′), 6.22 (s, 1H, H-3), 7.02 (m, 2H, H-6 + H-8), 7.80 (d, 1H, J = 8.7 Hz, H-5), 10.34 (s, 1H, NH), 10.53 (s, 1H, NH), 13 (s, 1H, OH). ^{13}C-NMR (75 ppm, DMSO-d_6): δ_c 18.2 (CH_3-9), 28.0 (CH_2-6′ + C-7′), 66.1 (CH_2-2′), 111.0 (CH-8), 112.8 (CH-3 + C-6), 114.2 (C-4a), 155.1 (C-8a), 123.9 (CH-5), 160.1–160.6 (C-2 + C-7), 165.9 (C-1′), 170.1 (C-5′), 173.5 (C-8′). ESI-HRMS: *m/z* $[M + H]^+$ calcd. for $(C_{16}H_{17}O_7N_2)^+$: 349.0958 found: 349.0964.

Biological activities

Antimicrobial evaluation

All the synthesized compounds have been evaluated for their antibacterial and antifungal activities. For antibacterial test, three bacterial agents were selected as test microorganisms, *Pseudomonas* sp. (Pa 499), *Burkholderia* sp. (Bg 35) and *Bacillus* sp. (Bp 420). They were cultured at 25 °C on Nutrient Agar (NA) medium for 48 h before use.

Antifungal activity was performed against *Aspergillus niger*, *Botrytis cinerea* and *Fusarium oxysporum* f. sp. *lycopersici*. These fungi were obtained from the Laboratory of Phytopathology of the Regional Center of Research in Horticulture and Organic Agriculture (CRRHAB) of Chott-Mariem, Tunisia. They were cultured at 25 °C on potato dextrose agar (PDA) medium 1 week before use. The screening results were compared with those of ampicillin and carbendazim used as standard references for antibacterial for antifungal activities, respectively.

Antibacterial activity

The purified products were screened for their antibacterial activity by using the agar disc diffusion method (Marmonier, 1987). NA medium cooled at 45 °C was supplemented with a bacterial suspension (10^6 CFU/mL) and poured into Petri plates. After solidification, sterile Whatman paper discs (diameter 6 mm) were placed at the surface of the culture medium and 20 µL (1000 µg/mL) of the product dissolved in DMSO was dropped onto each disc. The negative control plates had no product added to the filter paper, whereas in the positive control plates, discs were impregnated with the same volume of ampicillin solution (5 mg/mL). The treated Petri dishes were incubated at 25 °C for 48 h. The antibacterial activity was evaluated by measuring the diameter of the inhibitory zones formed around the discs. The experiment was replicated twice.

Antifungal activity

Aspergillus niger, *Botrytis cinerea* and *Fusarium oxysporum* f. sp. *lycopersici* were used for the screening of antifungal activity of the products tested by using the disc diffusion method (Barry and Thornsberry, 1991). A conidial suspension of the tested fungi was prepared (10^4–10^5 CFU/mL) and added to PDA medium cooled at 45 °C and poured uniformly into Petri plates (diameter 90 mm). Sterilized paper discs (6 mm, Whatman No. 1 filter paper) were impregnated with 20 µL (1000 µg/mL) of the product dissolved in DMSO and placed on the culture plates, whereas the negative control plates had no product added to the filter paper. In the positive control plates, discs were imbibed with the same volume of a carbendazim suspension (0.5 mg/mL). The diameter of the inhibition zone (mm) around the disc was measured after incubation at 25 °C for 4 days and compared with control. The test was performed in triplicate.

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