Synthesis of Coumarin Derivatives Coupled to Amino Acid Esters and Studying their Biological Activity as Antimicrobial Agents

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Abstract

A series of coumarin derivatives linked to amino acid ester side chains were synthesized and evaluated of their antibacterial and antifungal activity. The coumarin derivatives was alkylated by the ethyl bromoacetate and then using potassium carbonate to get alkylated hymecromone. Conventional solution method for amide bond formation was used as a coupling method between the carboxy-protected amino acids with acetic acid side chain of coumarin derivatives. The DCC/ HOBt coupling reagents were used for peptide bond formation. The proposed analogues were successfully synthesized and their structural formulas were consistent with the proposed structures as they were proved and characterized by thin layer chromatography (TLC), melting point, infrared spectroscopy (IR), and elemental microanalysis (CHN).

Key words: coumarin, amino acid, coumarin antimicrobialal activity.

تحضير مشتقات للكومارين مزدوجة مع استرات الاحماض الامينية مع دراسة للفعالية البايولوجية كمضاد للبكتريا

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الخلاصة

سلسلة من مشتقات الكومارين مرتبطة باسترات الاحماض الامينية قد تم تحضيرها ودراسة الفعالية الحيوية لها كمضادات بكتيرية ومضادات فطرية. مشتقات الكومارين قد تم ألكلتها بواسطة أثيل برومو حامض الخليك وباستخدام كاربونات البوتاسيوم للحصول على استر الهايميكرومون.إن تكوين اصرة الأميد تم باتباع طريقة المحلول التقليدية ما بين مشتقات الكومارين والتي تحتوي حامض الخليك كذراع جانبي مع حوامض امينية ذات مجموعات حامضية محمية إذ أن طريقة تخليق اصرة الببتيد باستخدام الاقتران المباشر بوجود DCC/HOBt والتي تتميز بكونها بسيطة وفعالة وتؤدي الى تحقيق عائد جيد عند درجة حرارة الغرفة. إن المركبات المقترحة تم تخليقها بنجاح إذ كانت الصيغ الهيكلية متسقة مع المركبات المقترحة بعد تمييزها وإثباتها باستخدام التقنيات التالية: كروماتوكرافيا الطبقة الرقيقة, مقياس درجة الانصهار , مطياف الاشعة تحت الحمراء, والتحليل الدقيق للعناصر المكونة.

الكلمات المفتاحية : كومارين, الاحماض الامينية, فعالية الكومارين كمضادات مايكروبية.

Introduction

Coumarins are heterocyclic compounds containing a lactone group. They are also known as benzo-2-pyrones. These compounds and their derivatives represent a broad class of natural and pharmaceutical products [(1)].This group of compounds has been isolated from a variety of plant sources. Coumarin was first isolated in 1820 by Vogel from the seeds of Tonka beans (coumarouna adorata) , while an umbelliferone (7-hydroxycoumarins) derivative present in many plants such as mannaash, sweet woodruff [(2)].Coumarin derivatives possess a wide range of pharmacological activities such as, anticoagulant [(3)], antifungal [(4)], antibacteria [(5)], anti-inflammatory [(6)], anticancer [(7)], spasmolytic and hypotensive activities [(8)]. 7-hydroxycoumarin is known for its antibiotic and antifungal activities. 8-Substituted-4-methyl-7-hydroxycoumarin [(9,10)] and 6-substituted-4-methyl-7-hydroxycoumarin [(11)] have been investigated for complexing ability. Hymecromone, (7-hydroxy-4-methyl-coumarin), is one of coumarin derivatives. It is used in liver therapy as a choleretic and spasmolytic with the special action on the gall ducts and gall bladder [(12,13)].Coumarins have attracted interest in recent years because of their diverse pharmacological properties [(14)] .During the last twenty years, the study of the biological activities of coumarin derivatives has been the aim of many researchers [(15)].

Chemical Synthesis

A. Esterification of amino acids

Synthesis of L-Leucine methyl ester HCl (Leu-O-Me), Compound (A.1) [(16)]

A suspension of Leucine (11 .4mmol, 1.5g) dissolved in (10ml) of absolute methanol, was cooled down to -15°C then thionyl chloride was added drop wise (11.4mmol, 0.86ml), (the temperature should

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be keep below –10°C), the reaction mixture was left at 40°C for 3hr, then refluxed for 3hr and left at room temperature overnight. The solvent was evaporated to dryness under vacuum, re-dissolved in methanol and evaporated, this process was repeated several times and re-crystallize the product from methanol-diethyl ether. Percent yield, physical appearance, m.p, R_f values were listed in table (1), and IR characteristics absorption bands were listed in table (2).

Synthesis of methionine methyl ester HCl (Met -O-Me), Compound (A.2).

A suspension of Methionine (10mmol, 1.5g) dissolved in (15ml) of absolute methanol and (10mmol, 0.75ml) of thionyl chloride, then complete the procedure as mentioned in the synthesis of A.1. Percent yield, physical appearance, m.p, R_f values were listed in table (1), and IR characteristics absorption bands were listed in table (2).

B. Alkylationof 4-methyl-7-hydroxycoumarin Compound (B) [17]

A suspension of 4-methyl-7-hydroxy coumarin (39.7mmol, 7g) in acetone (70ml) was refluxed with ethyl bromoacetate (59.55mmol, 6.5ml) and anhydrous K_2CO_3 (218.3mmol, 30g) for 16 hr. after cooling, the mixture was evaporated to dryness. The residue was recrystallized from acetone to give off-white powder of 4-methyl-7-(methoxy ethyl acetate) coumarin.Percent yield, physical appearance, m.p, R_f values were listed in table (1), and IR characteristics absorption bands were listed in table (2).

C. Synthesis of 4-methyl-7-(carboxy methoxy) coumarin, Compound (C) [18]

Compound (B) (26.7mmol, 7g) was dissolved in (100ml) of absolute ethanol and (42ml) of 5% NaOH solution was added, the reaction mixture was stirred overnight at room temperature, then the solvent evaporated, and the residual was dissolved in water and acidified with 6 N of HCl. The white precipitate was filtered and crystallized from ethanol. Percent yield, physical appearance, m.p, R_f values were listed in table (1), and IR characteristics absorption bands were listed in table (2).

D. Coupling method and reagents

Conventional solution method was used as a coupling method between the carboxy- protected amino acid and carboxylic acid of coumarin derivatives. DCC (Dicyclohexyl carbodiimide) was used as a coupling reagent, while HOBt (1-Hydroxy benzotriazole) was used to decrease racemization and to increase the yields.[19]

1) Synthesis of 4-methyl coumarin-7-O-acetyl - leucine methyl ester, Compound (D.1) [20]

To a stirred solution of leucine methyl ester HCl (compound A.1) (1.96mmol, 0.357g) in (6ml) of DMF, (1.96mmol, 0.2ml) of N-methyl morpholine (NMM) was added with stirring for 10 min., then (1.96mmol, 0.45 g) of (comp C) was also added, and the mixture was cooled down to (-10°C) then (3.9mmol, 0.62g) of HOBt and (1.96mmol, 0.4g) of DCC were added with stirring, which was continued for 2days at 0°C and then at room temperature for 6 days. The crude product was evaporated to exclude DMF and redissolved in chloroform from which the N,N–Dicyclohexyl urea (DCU) was filtered off , and the clear filtrate washed twice with 5% sodium carbonate solution, 0.1N HCl, once with water, and with saturated sodium chloride solution.The chloroform layer was dried with anhydrous magnesium sulfate and evaporated under vacuum; the resulted product was collected, recrystallized from (methanol: chloroform) (5:1). Percent yield, physical appearance, m.p, R_f values were listed in table (1), IR characteristics absorption bands were listed in table (2) and elemental microanalysis data are listed in table(3).

2) Synthesis of 4-methyl coumarin-7-O-acetyl - methionine methyl ester, Compound (D.2)

To a stirred solution of methionine methyl ester HCl (compound A.2) (2.5mmol, 0.5g) in (7.5ml) of DMF, (2.5mmol, 0.27ml) of N-methyl morpholine (NMM) was added with stirring for 10 min., then (2.5mmol, 0.58 g) of (comp C) was also added, and the mixture was cooled down to (-10°C) then (5mmol, 0.8g) of HOBt and (2.5mmol, 0.5g) of DCC were added with stirring, which was continued for 2days at 0°C and then at room temperature for 6 days. Then the procedure was completed as mentioned in the synthesis of D.1. Percent yield, physical appearance, m.p, R_f values were listed in table (1), IR characteristics absorption bands were listed in table (2) and elemental microanalysis data are listed in table(3).

Table 1 : The physical appearance, percent yield, melting points and R_f values of the synthesized compounds and their intermediates.

Compound	Physical Appearance	%Yield	Melting Point Observed (^oC)	R_f Value	Solvent system
A.1	White needle shape crystals	91	146-148	0.78	Chloroform 7 Methanol 3
A.2	White needle shape crystals	90	148-150	0.8	Chloroform 7 Methanol 3
B	Off white powder	74	96-97	0.7	Chloroform 4 Methanol 6
C	Off white crystals	79	207-208	0.8	Chloroform 2.5 Methanol 2.5 Ether 5
D.1	White crystals	92	103-105	0.74^D	Chloroform 2 Methanol 8
D.2	Off white to yellow crystals	89	105-108	0.76^D	Chloroform 2 Methanol 8

Table 2 : IR characteristic absorption bands.

Comp. No.	Compound Name	IR Characteristic Absorption Bands (V cm-1)
A.1	Leu -O-Me	(3032-2784 NH stretch of amine salt), (2958, 2924, 2872 assym. str.CH_3,CH_2, CH), (1739 C=O Ester),(1508 NH bend), (1451 CH_3,CH_2 bend), (1394,1359 CH bend) , (1228 C-O Ester).
A.2	Meth-O-Me	(3136-2857 NH stretch of amine salt), (2956, 2914 assym. str. CH_3 , CH_2), (1747 C=O of Ester) , (1489 NH bend), (1444 CH_3 bend), (1232 C-O of Ester).
B	4-methyl-7-(methoxy ethyl acetate)coumarin	(3076 CH str. of aromatic C=CH), (2980, 2928 assym. str. CH_3, CH_2), (2872 sym. str. CH_3), (1759 C=O Ester), (1606, 1508 str. aromatic C=C), (1423, 1386 CH_3, CH_2 bend), (1220 C-O Ester), (1197 C-O Lacton).
C	7-(carboxy methoxy)-4-methyl coumarin	(3200-2500 str.OH), (3068 CH str. of aromatic C=CH), (2987, 2916 assym. str. CH_3, CH_2), (1755 C=O Carboxylic acid), (1708 C=O Lacton), (1610, 1566, 1510 str. aromatic C=C), (1427, 1390 CH_3, CH_2 bend), (1253 C-O carboxylic acid), (1147 C-O Lacton).
D.1	4-methyl-7-O-acetyl-leucine methyl ester	(3333 NH stretch, amide II), (2955,2928, 2852 assy. str. CH_3,CH_2, CH), (1739 C=O of Ester and Lacton), (1676 C=O str. amide), (1620 C= C aromatic), (1527 NH bend, amide II), (1390,1369 CH bend), (1298 C-O Ester), (1151 C-O Ether).
D.2	4-methyl-7-O-acetyl-methionine methyl ester	(3362 NH stretch, amide II), (2926, 2852 assy., sym str. CH_3,CH_2), (1741 C=O of Ester and Lacton), (1670 C=O str. amide), (1622 C= C aromatic), (1527 NH bend, amide II), (1440,1390 CH_3,CH_2 bend), (1300 C-O Ester), (1155 C-O Ether).

Table 3 : The elemental microanalysis % of final products

Compound	Value type	C	H	N	O	S	Mol.wt.
D1	calculated observed	63.15 64.63	6.41 6.60	3.88 4.07	26.56 27.31		361.39
D2	calculated observed	56.98 58.03	5.58 5.39	3.69 3.81	25.30 26.10	8.45 8.78	379.43

Biological Activity

A preliminary antibacterial and antifungal activity has been carried out according to Well Diffusion Method:

The prepared compounds have been studyied for their antimicrobial activity in vitro against three tested bacteria (*Staphylococcus aureus.*, *Streptococcus spp.* as gram positive bacteria *and proteus spp*. as gram negative bacteria) and two fungi (*Aspergillus spp., and Candida spp*.) were clinical activated and maintained on nutrient agar medium for testing antibacterial activity and sabaroud agar medium for antifungal activity.Ofloxacin was used as a standard drug for antibacterial activity and Ketoconazole was used as a standard drug for antifungal activity.The plates were incubated at 30 °C for 72 hours (fungai spp.) or 37 °C for 24 hours (bacteria) [21] and the antimicrobial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the disc in mm, as show in table (4) and (5) respectively .

Result and Discussion

Synthetic part

The overall synthesis strategy based one four major lines:

1. Amino acid derivatives

The amino acids were activated by thionyl chloride to get acyl chloride that attacks methanol to get methyl esters of the selected amino acids.

2. Alkylation of hymecromone

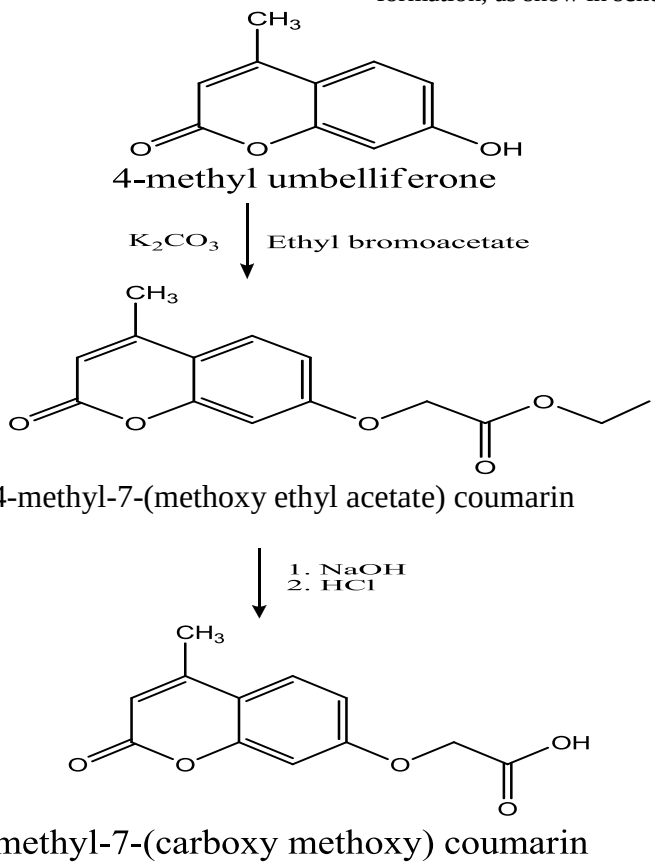
Hymecromone was alkylated by the ethyl bromoacetate and then using potassium carbonate to get ester of hymecromone.

3. Hymecromone acetic acid synthesis

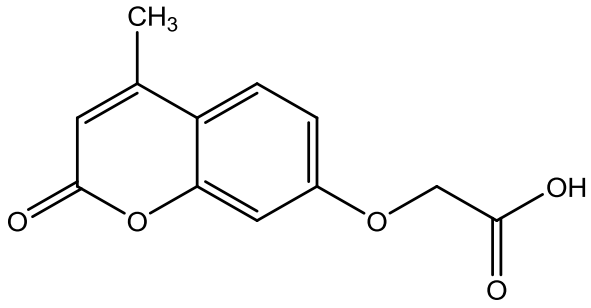
Removal of ethoxy group from the hymecromone ester can be done by sodium hydroxide to form the conjugated base, then acidified with hydrochloric acid to get hymecromone acetic acid, as show in scheme (1).

4. Amide bond formation

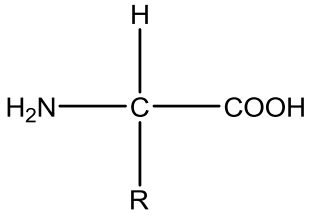
Conventional solution method for amide bond formation used as a coupling method between the carboxy-protected amino acids with acetic acid side chain of hymecromone. The DCC/ HOBt coupling reagents used for peptide bond formation, as show in scheme (2).



Scheme 1 : Pathway of synthesis of 4-methyl coumarin carboxylic acid.

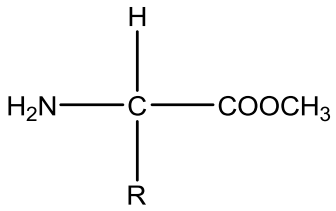


7-(carboxy methoxy)-4-methyl coumarin



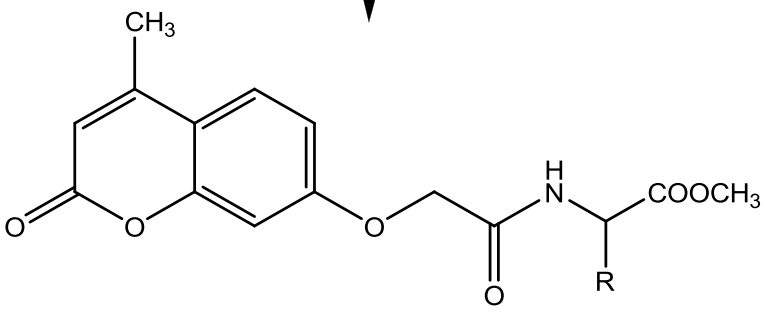
Amino acid

carboxyl protection



Amino acid ester

DCC
HOBt
coupling
reaction



R : is alkyl group

D1: R= $CH_2 CH (CH_3)_2$
D2: R= $(CH_2)_2 S CH_3$

Scheme 2 : Pathway of synthesis of coumarin derivative coupled to amino acid esters.

nutrient agar medium for testing antibacterial activity and sabaroud agar medium for antifungal activity.Ofloxacin was used as a standard drug for antibacterial activity and Ketoconazole was used as a standard drug for antifungal activity.The plates were incubated at 30 °C for 72 hours (fungai spp.) or 37 °C for 24 hours (bacteria) [21] and the antimicrobial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the disc in mm, as show in table (4) and (5).

Table 4 : The antibacterial activity of the tested compounds

Compound no.		Zone of Inhibition in mm		
		Staphylococcus aureus	***Streptococcus spp.***	***Proteus spp.***
D1	2µg/ml	5	3	3
	20µg/ml	9	10	8
	50µg/ml	14	12	11
D2	2µg/ml	1	2	No activity
	20µg/ml	4	3	No activity
	50µg/ml	7	7	No activity
Ofloxacin	2µg/ml	5	6	5
	20µg/ml	10	12	10
	50µg/ml	16	17	16

Table 5: The antifungal activity of the tested compounds.

Compound no.		Zone of Inhibition in mm	
		Aspergillus spp.	***Candidia spp.***
D1	5µg/ml	6	7
	20µg/ml	13	12
	50µg/ml	14	18
D2	5µg/ml	3	7
	20µg/ml	6	10
	50µg/ml	12	14
Ketoconazole	5µg/ml	12	9
	20µg/ml	17	25
	50µg/ml	20	30

The antimicrobial activities (antibacterial and antifungal activities) have been carried out according using Well Diffusion methodology.The prepared compounds have been studying for their antibacterial activity *in vitro* against three tested bacteria (*Staphylococcus aureus., Streptococcus spp.* as gram positive bacteria *and proteus spp*. as gram negative bacteria) respectively using Ofloxacin as standard. Also, the fungal activity has been study against two types of fungi (*Aspergillus spp., and Candida spp*.) using Ketoconazole as standard.All results are fixed in tables (4) and (5), compound D1 show antibacterial activity, while both compounds D1&D2 have a good antifungal activity.

Conclusion

The proposed compounds were successfully synthesized by the conventional solution method as previously described and their structure formula were consistent with the proposed structures since conformity of their structures was achieved by using the following techniques: thin layer chromatography (TLC), melting point, infrared spectroscopy (IR) and elemental microanalysis (CHN).The best activity of the studied samples appeared in the compounds D1 (4-methyl coumarin-7-O-acetyl-leucine methyl ester) as antibacterial

agent, and antifungal agent.The mechanism of their action is not yet fully understood and correlation of effects with chemical structures is not conclusive at the moment.

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