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Synthesis and anticoagulant activity of new small molecules containing sulfated saccharides

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Abstract

This work reports the synthesis and anticoagulant activity of new small molecules containing sulfated saccharides. Heparin, which is a polysulfated polysaccharide, is a short anticoagulant acting agent with an effective mechanism of action involving several plasmatic factors of the coagulation, namely thrombin, factor Xa (FXa), and platelets factors. Unfortunately, heparin is a heterogeneous product obtained from bovine or porcine sources, active only by parental route. In opposite, coumarin-derivatives possessing a 4-hydroxyl group have been therapeutically used for their oral bioavailability (e.g.warfarin). Nevertheless, being vitamin K antagonists, coumarins have several interactions with many drugs and food. New oral anticoagulants were introduced in therapy in the last decade, acting by direct inhibition of FXa and thrombin, which have provided impressive clinical results in the trials for the prophylaxis of postoperative venous thrombosis. However, safety concerns related to liver enzymes increase and bleeding risks associated with their prolonged use have been described, adding to their high costs.

In this work, an hybridization strategy was planned to develop new anticoagulant agents: obtain new coumarin derivatives with a persulfated saccharidic moiety and an heterocyclic linker. Seven coumarins derivatives were synthesized: two propargylated coumarins, three acetylated glycosyl triazole coumarins, one deacetylated glycosyl triazole coumarin, and one new persulfated glycosyl triazole coumarin. From the synthesized derivatives, four are new compounds. The structure elucidation of the synthesized coumarins was established by IR, ^{1}H, ^{13}C NMR and HSQC/HMBC techniques.

The anticoagulant activity of the new sulfated coumarins as well as of another sulfated compound previously synthesized in LQOF (persulfated glycosyl ascorbic acid), was measured in human plasma by the three classical clotting times – activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) – in five different concentrations. All tested compounds showed anticoagulant properties in a dose dependent manner. The APTT assay was the most sensitive test to the presence of tested sulfated compounds. Only sulfated 7-substituted glycosyl heterocycle coumarin linked was found to double three clotting times assays. The most promising sulfated compounds were both 7- cellobiosyl coumarin (with and without the heterocyclic linker) ($APTT_2$ = ± 200 µM) and sulfated glycosyl ascorbic acid ($APTT_2$ = ± 130 µM).

Resumo

Este trabalho retrata a síntese e atividade anticoagulante de novas pequenas moléculas contendo sacarídeos sulfatados.

A heparina, que é um polissacarídeo polissulfatado, é um agente anticoagulante de curta duração com um mecanismo de ação efetivo envolvendo vários fatores plasmáticos da coagulação, como a trombina, o fator Xa (FXa) e outros fatores plaquetários. Infelizmente, a heparina é um produto heterogéneo obtido a partir de fontes bovinas ou suínas, ativo apenas por via parenteral. Em contrapartida, os derivados de cumarina que possuem um grupo 4-hidroxilo foram utilizados na terapêutica e possuem biodisponibilidade oral (por exemplo, a varfarina). No entanto, sendo antagonistas da vitamina K, as cumarinas têm várias interações com muitos medicamentos e alimentos. Na última década, foram introduzidos na terapêutica novos anticoagulantes orais, atuando por inibição direta do FXa e da trombina, os quais proporcionaram resultados clínicos impressionantes nos ensaios para a profilaxia da trombose venosa pós-operatória. No entanto, surgem preocupações de segurança relacionadas ao aumento de enzimas hepáticas e riscos de sangramento associados com o seu uso prolongado.

Neste trabalho, foi planeada uma estratégia de hibridização para desenvolver novos agentes anticoagulantes: obter novos derivados cumarínicos com uma porção sacarídica persulfada e uma ponte heterocíclica. Foram sintetizados seis derivados de cumarinas: três acetilglicosídeos, um glicosídeo e uma nova cumarina glicosídica sulfatada. Dos derivados sintetizados, quatro são novos compostos. a elucidação estrutural das cumarinas sintetizadas foi estabelecida pelas técnicas de IV, RMN de ^{1}H e de ^{13}C e HSQC / HMBC.

A atividade anticoagulante das novas cumarinas sulfatadas bem como de outro composto sulfatado previamente sintetizado no LQOF (glicosideo de ácido ascórbico persulfatado) foi medida em plasma humano pelos três tempos de coagulação clássicos - tempo de tromboplastina parcial activada (APTT), tempo de protrombina (PT) e tempo de trombina (TT) - em cinco concentrações diferentes. Todos os compostos testados mostraram propriedades anticoagulantes de uma maneira dependente da dose. O ensaio APTT foi o teste mais sensível à presença de compostos sulfatados testados. Verificou-se que apenas a 7-celobiosil cumarina com triazole duplicava os três ensaios de tempos de coagulação. Os compostos sulfatados mais promissores foram a cumarina substituída com celobiose na posição 7 (com e sem anel heterocíclico) ($APTT_2$ = ± 200 μM) e o ácido ascórbico glicosilado sulfatado ($APTT_2$ = ± 130 μM).

Abbreviations

^{13}C NMR – Carbon nuclear magnetic resonance

^{1}H NMR – Proton nuclear magnetic resonance

APTT – Activated partial thromboplastin time

AT III – Antithrombin III

CT – Clotting time

CuAAC – Copper (I)-catalyzed azide-alkyne cycloaddition

d - Doublet

dd – Double doublet

DMA – Dimethylacetamide

DMSO-d_6 - Hexadeuterodimethyl sulfoxide

Eq. – Equivalent

h – Hours

HIT - Heparin-induced thrombocytopenia

HSQC - Heteronuclear single quantum coherence spectroscopy

HMBC - Heteronuclear multiple bond correlation

IR – Infrared spectroscopy

J – Coupling constant

LMWHs - Low-molecular-weight heparins

LQOF – Laboratório de química orgânica e farmacêutica

m – Multiplet

M.p. – Melting point

min. – Minutes

MW – Microwave

NA – Not active

NMR – Nuclear Magnetic Resonance

PT – Prothrombin time

r.t. – Room temperature

s – Singlet

SPE – Solid phase extraction

t – Triplet

TBAB – Tetra-*n*-butylammonium bromide

TEA – Triethylamine

TEA:SO_3 – Triethylamine sulfur trioxide adduct

TF – Tissue factor

TFPI - Tissue factor pathway inhibitor

THF – Tetrahydrofuran

TLC – Thin layer chromatography

TT – Thrombin time

UFHs - Unfractionated heparins

VKAs – Vitamin K-antagonists

Structure and organization of this dissertation

Chapter 1 – Introduction

In chapter 1, a brief explanation about the physiology of coagulation and the anticoagulant therapy is presented. In this section, the methods used to evaluate the plasmatic blood coagulation are also described. References are listed at the end of each chapter.

Chapter 2 – Original article: Hybrids of sulfated glycosides and coumarins as new anticoagulant agents

Chapter 2 integrates an original article in preparation. This original work includes the synthesis of sulfated glycosyl coumarins and the evaluation of the anticoagulant activity.

Chapter 3 – Other sulfated small molecules containing saccharides

This chapter presents the results and discussion of the synthesis, structural elucidation as well as the material and methods of coumarins, 6-cellobyl and 4-glycosyl.

Chapter 4 - Anticoagulant activity evaluation of other sulfated compounds

This chapter describes the evaluation of the anticoagulant activity of another sulfated compound previously synthesized in the Laboratory of Organic and Pharmaceutical Chemistry (LQOF).

Chapter 5 – Conclusion

In this chapter, a general conclusion is presented, and future perspectives are given.

General and specific objectives

The general aim of this dissertation was the synthesis of new coumarin derivatives with a polysufated saccharidic moiety.

The specific aims include:

- synthesis of building blocks;

- synthesis of 4-, 6- and 7- glycosyl coumarins;

- synthesis of persulfated 6- and 7- glycosyl coumarins;

- evaluation of the anticoagulant activity of the synthesized sulfated coumarins and of another related sulfated compound available in LQOF.

Chapter 1 – Introduction

1. Introduction

New molecules have been implicated in the pathophysiology of venous thromboembolism and are now the target of studies that may bring new forms of prevention, diagnosis and treatment. The term thrombosis refers to the formation, from constituents of blood, of an abnormal mass within the vascular system of a living animal. When this process occurs within deep veins, it is referred to as deep vein thrombosis.

1.1. Hemostasis

Blood coagulation, also called secondary hemostasis, is based on a complex enzymatic cascade in which the components are activated by proteolysis and whose product is fibrin that forms the base of the blood clot.[1] The coagulation process (**Figure 1.1**) results from the sequential activation of a series of interacting plasma proteins and the formation of membrane complexes with the calcium ions. These proteins are called coagulation factors and circulate in plasma in their inactive forms (zymogens).[2]

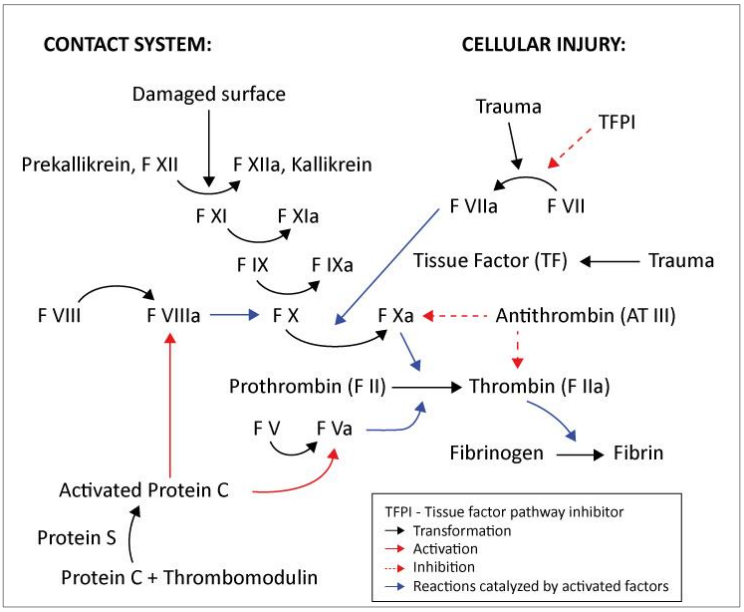

Figure 1.1. Coagulation cascade.

There are mechanisms that oppose the formation of the clot to limit the process and prevent it from spreading excessively. The failures in these mechanisms of hemostasis regulation are associated with hypercoagulability states.

The most relevant physiological anticoagulants are: the tissue factor pathway inhibitor (TFPI), antithrombin III (AT III), protein C, and protein S. (**Figure 1.1**). The anticoagulant mechanism that acts at on the earliest stage of coagulation is TFPI, which binds to the TF/FVIIa complex inhibiting its ability to act on factor X (FX).[3] AT III is the main circulating inhibitor of coagulation, directly inactivating thrombin as well as other serine proteases (factors IXa, Xa, XIa, XIIa), plasmin and kallikrein. Protein C is activated by thrombin after binding to thrombomodulin (present on intact endothelial surfaces). Activated protein C inactivates factors Va and VIIIa as well as platelet receptors for FXa. Protein S, like protein C, is a vitamin K dependent protein and its free form acts as a cofactor of activated protein C along with procoagulant phospholipids (**Figure 1.1**).[3]

1.2. Unfractionated heparins, low molecular weight heparins and fondaparinux

Unfractionated heparins (UFHs), were discovered in the beginning of the last century.[4] This natural sulfated linear glycosaminoglycan (**Figure 1.2**) is produced by the mast cells of several animal tissues.[5]

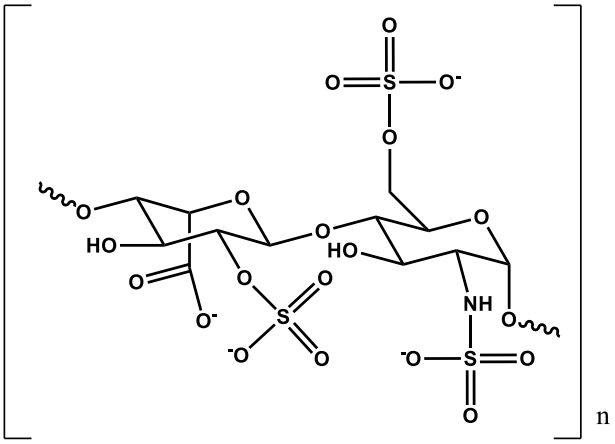

Figure 1.2. Structure of heparin.

UFH is activated in the bloodstream only in the presence of one of the players of this cycle, AT III, via complexing with it. The binding site is a pentasaccharide fragment with a specific sequence of sugar residues in the heparin chain the formed heparin–AT III complex inactivates thrombin, which is accompanied by a drastic increase in the AT III inhibitory effect on the other proteinases involved in blood clotting.

In the last decades the introduction of several low molecular weight heparins (LMWHs) have gained greater acceptance for a number of indications. LMWHs consist of only short chains of polysaccharide. These are obtained by various methods of fractionation

or depolymerization of polymeric heparin. These derivatives correspond to a decrease in molecular weight of about one third of the UFHs. Therefore, at least half of the LMWHs chains are too short to bind both thrombin and ATIII. Thus, LMWHs have a lower activity against thrombin than UFHs. LMWHs are characterized in low doses because they have a higher bioavailability, a longer half-life and a more predictable dose-response effect than UFH. The risk of developing hemorrhagic complications and incidence of HIT in response to LMWH therapy is decreased although there is still there.[6]

The pharmacokinetic and biological advantages of LMWHs relative to UFHs have stimulated interest in fragments of still lower UFHs. The discovery in the 80`s that a domain of only five residues in UFHs activated AT III to accelerate the inhibition of FXa, but not thrombin, led to the synthesis of a single pentasaccharide sequence, called fondaparinux The need for subcutaneous parenteral administration[7] makes fondaparinux an inconvenient and expensive drug. The biological action of the active pentasaccharide fondaparinux, stems on the active pentassacharide concept that it represents the essential heparin sequence that strongly binds to AT III and dramatically increases the rate of AT III mediated inhibition of FXa while only marginally inhibiting thrombin.[8-9] In patients suffering from heparin-induced thrombocytopenia (HIT) fondaparinux have been used.[10]

Heparin is involved beyond the coagulation cascade,[11] showing antimetastatic[12] and antinflammatory activities[13] which confers a polypharmacological mode of action. Heparins do not exhibit oral bioavailability due to their highly negative charge, large molecular weight[14] and rapid metabolism in gastrointestinal tract.[15] The rapid metabolism tract is mainly due to low stability at low pH[16] and the enzymatic degradation by intestinal microflora.[17-18] Parenteral administration of heparins is a costly and invasive method that leads to low patient adherence.

1.3. Vitamin K-antagonists

Vitamin K-antagonists (VKAs) are widely prescribed for treatment of thromboembolic events, but these medications have a narrow therapeutic index, indicated by the fact that administration and use of these drugs are associated with clot formation and bleeding when the dose is subtherapeutic and supratherapeutic, respectively. Therefore, dosage adjustments are frequently required as there is a large inter-individual variation in drug response. This is especially important in the initial phases of oral-anticoagulant therapy.[19] VKAs are oral availability due to the hydrophobic character that the coumarin

nucleus offers; however, an extensive list of drug and food interactions strongly limits their use.

Commercially available coumarins include warfarin, acenocoumarol, phenprocoumon, and dicoumarol (**Figure 1.3**).

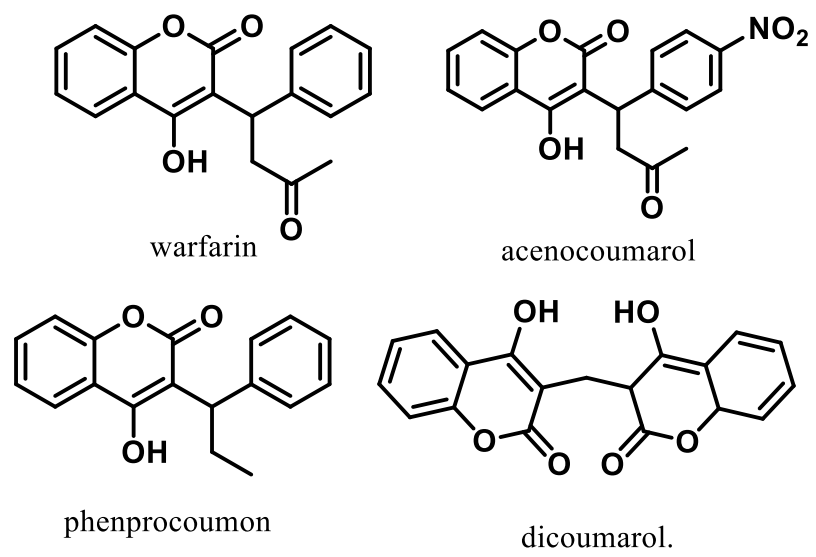

Figure 1.3. The vitamin K-antagonists structures.

Warfarin began to be used in clinical practice at a time when there were other anticoagulants such as heparin and dicumarol itself, but these had disadvantages: heparin could only be used parenterally and dicumarol had a long latency period before beginning to take effect. Thus, the main advantages of warfarin were high solubility in water and high oral bioavailability,[20-21] however, the monitoring problem remained.

Vitamin K acts as a cofactor for the carboxylation of specific residues of glutamic acid to form γ-carboxyglutamic acid an amino acid present in coagulation factors (factors II, VII, IX and X). In order for the factors II, VII, IX, X, C and S proteins to become active, the γ-carboxylation of glutamic acid must occur, thus allowing the adhesion of these proteins to the surface phospholipids, accelerating the coagulation process[22] (**Figure 1.4**). Vitamin K epoxide reductase recycles reduced vitamin K, which is used subsequently as a co-factor in γ-glutamyl carboxylase residues in blood coagulation enzymes. Availability of vitamin K for this reaction requires prior reduction of vitamin K epoxide by vitamin K epoxide reductase in the endoplasmic reticulum membrane.[19] The vitamin K-dependent γ-carboxylation system consists of the vitamin K-dependent γ-carboxylase, which requires the reduced hydroquinone form of vitamin K a cofactor and the warfarin-sensitive enzyme vitamin K

epoxide reductase, which produces the cofactor.[23] Concomitant with γ-carboxylation, the vitamin K hydroquinone is converted to the vitamin K epoxide metabolite[19] (**Figure 1.4**).

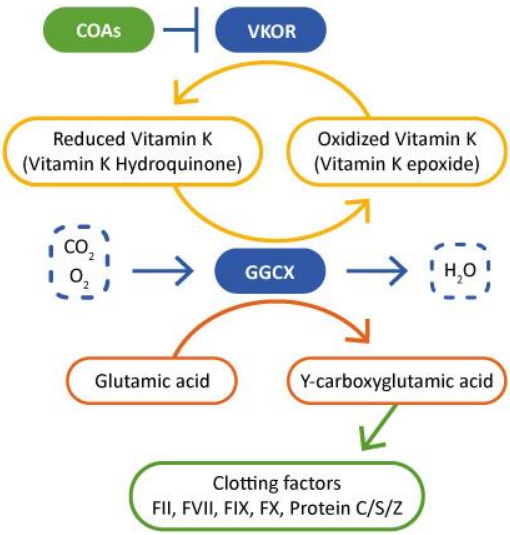

Figure 1.4. Vitamin K cycle and action of vitamin K-antagonists (or coumarinic oral anticoagulants). Vitamin K-antagonists inhibit the VKOR, enzyme involved in the regeneration of oxidized form of vitamin K. This indirectly stops the production of biologically active forms of clotting factors II, VII, IX, X and proteins C/S/Z. COAs: coumarinic oral anticoagulants; VKOR: vitamin K epoxide reductase; GGCX: γ-glutamyl carboxylase; → catalytic action; and ⊣ inhibitory action. Adapted from.[19]

This results in the inability of the coagulation factors to get carboxylated at glutamic acid residues rendering them biologically inactive. This interconversion of vitamin K metabolites is known as the vitamin K cycle (**Figure 1.4**). Patients suffer excessive bleeding if they are unable to recycle vitamin K from vitamin K epoxide.[19]

1.4. New oral anticoagulants

The new oral anticoagulant drugs acting by direct FXa-inhibition (edoxaban, apixaban and rivaroxaban) (**Figure 1.5**) possess the heteroaromatic ring in their structure and it was observed that, heteroaromatic rings would be good neutral motifs for binding to the FXa protein as inhibitors.[24] These FXa-inhibitors anticoagulant drugs are basic and more lipophilic than inhibitors with highly basic residues and have a greater ability to pass through the lipophilic epithelia of the gastrointestinal tract. Dabigatran (**Figure 1.5**) is a direct thrombin inhibitor that reversibly binds to the active site of thrombin.

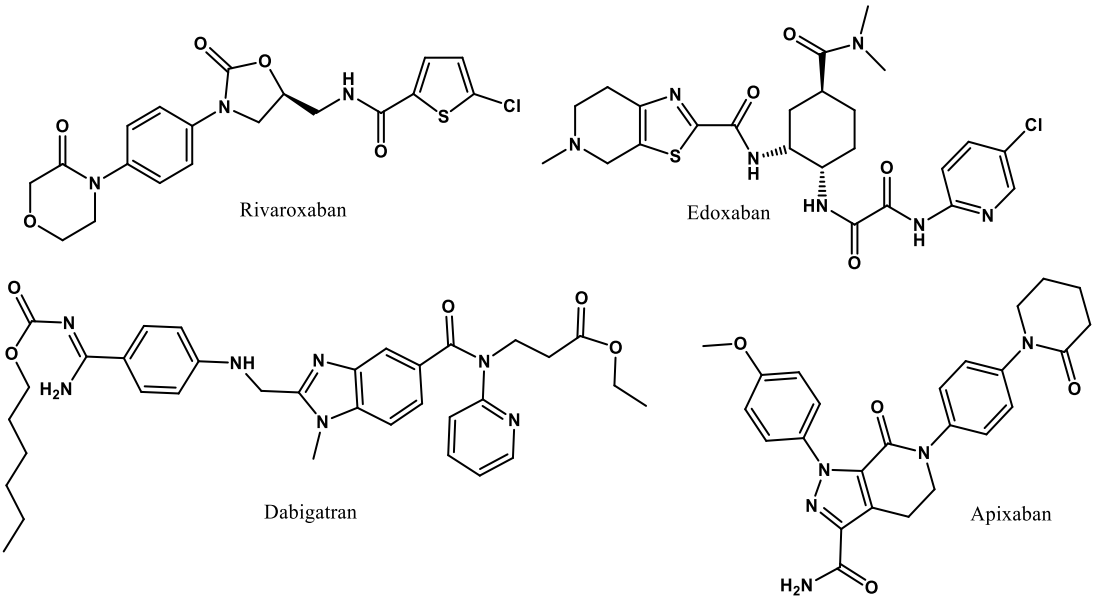

Figure 1.5. Structures of new oral anticoagulants.

Oral direct thrombin and FXa inhibitors offer many advantages over vitamin K-antagonists and parenteral anticoagulants for several common clinical indications.[25-26] Selective FXa inhibitors compared with direct thrombin inhibitors have been shown to decrease endogenous thrombin potential.[27-29] Compared with thrombin, FXa has limited functions other than serving as the principal mediator of thrombin generation from prothrombin via the prothrombinase complex.[30] FXa does exhibit proinflammatory and proliferative activities, but thrombin has a far greater number of important activities within and outside of the hemostatic system,[31-32] the inhibition of which might influence the efficacy and safety of specific thrombin inhibitors. Perhaps of greatest importance in this regard is thrombin prothrombotic role in platelet activation, thrombin positive feedback on coagulation factors earlier in the coagulation figure, and thrombin antithrombotic role in the activation of protein C and thrombin activatable fibrinolysis inhibitor. Thrombin is the principal activator of platelets at sites of injury through its interaction with platelet protease activated (PAR) 1 and 4 receptors[33-34] whereas FXa has no effect on platelet activation.[27] Selective FXa inhibitors compared with direct thrombin inhibitors have been shown to decrease endogenous thrombin potential.[27-29]

1.5. Evaluation of coagulation - classical clotting times

The influence of compounds on the plasmatic blood coagulation is usually determined by measuring the activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT), which allow the evaluation of the ability to inhibit blood

clotting through the intrinsic, extrinsic, and common pathways of the coagulation cascade, respectively.[35]

The PT assay is the time taken to fibrin strand formation when platelet-poor plasma is recalcified in the presence of thromboplastin (tissue factor and phospholipid). Considered a specific test for a deficiency of the extrinsic pathway, this test is most sensitive to a decrease in FVII however, it is also prolonged with deficiencies of FI, FII, FV, and FX (**Table 1.1**), and in liver disease, vitamin K deficiency (by inhibiting the reduction reactions in the vitamin K cycle and decreasing the synthesis of vitamin K–dependent coagulation factors) and by high doses of heparin.[36-37]

Table 1.1. Coagulation factors enrolled in each clotting time.[38]

	APTT (intrinsic pathway)	PT (extrinsic pathway)	TT (common pathway)
Coagulation factors	kininogen, prekallikrein, I, II, V, VIII, IX, X, XI, and XII	I, II, V, VII, and X	V, X, II, I and II

The APTT in contrast to the PT measures the activity of the intrinsic pathways of coagulation and is used to determine the function and integrity of the final common pathway; particularly, the coagulation factors kininogen, prekallikrein, XII, XI, IX, VIII, X, V, I and II are investigated (**Table 1.1**). In tests *ex-vivo*, the various APTT reagents contain phospholipids and a specific contact activator. The APTT is frequently used to monitor patients receiving unfractionated heparin. Vitamin K antagonists as warfarin prolonged the APTT only by a few seconds in patients with a stable target INR; in patients who are overdosed, the APTT may be significantly prolonged.[37, 39] A silicate or other chemical reagent is used to activate plasma contact-activation factors in combination with phospholipids and calcium to partially activate FXa. This leads to the formation of small amounts of thrombin, which activate FXI and FVIII. The FXIa and the FVIIIa/IXa/calcium complex activates FX. FXa along with factor V converts prothrombin to thrombin, and thrombin cleaves fibrinogen to form the fibrin clot. This process generally takes 30 to 35 s, depending on the reagents used. Thus, the APTT is sensitive to the levels of every clotting factor except factor VII (**Table 1.1**).[40]

The TT involves only the addition of bovine or human thrombin to platelet poor plasma. It reflects the conversion of fibrinogen to fibrin (**Table 1.1**). Heparin produces a dose-dependent prolongation of TT. Factor Xa inhibitors or vitamin K antagonists as warfarin have no impact on the TT assay.[39]

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Chapter 2 - Original Article: Hybrids of sulfated glycosides and coumarins as new anticoagulant agents

2. Original Article: Hybrids of sulfated glycosides and coumarins as new anticoagulant agents

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Abstract: Due to the limitations of anticoagulants in therapy, the search for new anticoagulant agents is still a medical need. In this work, an hybridization strategy was planned: obtain new coumarin derivatives with a persulfated glycosidic moiety and a heterocycle linker. Twelve new coumarin derivatives were synthesized: two propargylated derivatives, three acetyl glycosylated, three glycosylated, and four new sulfated coumarins. The synthesized coumarins were fully characterized by IR, ^{1}H NMR, ^{13}C NMR and HSQC/HMBC techniques. The anticoagulant activity was measured by the three classical clotting times, activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT). The four sulfated coumarins (**6**, **10**, **12**, and **17**) were found to prolong the clotting time in a dose-dependent manner. Sulfated coumarin **10** was able to prolong the three clotting times ($APTT_2$ = 216 μM, PT_2 = 812 μM and TT_2 = 1863 μM), while sulfated coumarin **12** was only able to prolong $APTT_2$ = 222 μM and PT_2 = 2439 μM). Sulfated coumarins **6** and **7** only affected the APTT assay with higher potency values.

Keywords: anticoagulants; coumarins; glycosylation; sulfation

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2.1. Introduction

Over the last 15 years, according to the World Health Organization the world's leading causes of death are ischemic heart disease and stroke, which constitute the number

one and two of the top 10 global causes of death and accounted for a combined 15.2 million deaths in 2016.[1-2]

Heparin has been used since the beginning of the last century.[3] Heparin is a natural sulfated linear glycosaminoglycan produced from animal tissues.[4] Because of its high efficiency, heparin has become the most frequently used drug in vascular surgery for prevention of clotting during long interventions and postoperative thromboses. The APTT increases soon after administration of heparin[5] by acting with an effective mechanism of action involving inhibition of FXa and thrombin by AT III activation.[3] A wide administration of this anticoagulant in medical practice has also demonstrated several adverse side effects such as heparin induced thrombocytopenia (HIT) and hemorrhagic complications. The heparin being a polysaccharide has no oral availability and is therefore administered intravenously which is costly and invasive.

Warfarin is vitamin K antagonists (VKAs) and it is widely prescribed for treatment of thromboembolic events for long periods of treatment. This is especially important in the initial phases of oral-anticoagulant therapy[6] while heparin is used for short-term treatments.[7] VKAs medications have a narrow therapeutic index, indicated by the fact that administration and use of these VKAs are associated with clot formation and bleeding when the dose is subtherapeutic and supratherapeutic, respectively. Therefore, dosage adjustments are frequently required as there is a large inter-individual variation in drug response. Furthermore, being VKAs, coumarins have a delayed onset and offset of action and an extensive list of drug and food interactions. The prothrombin time (PT), expressed as international normalized ratio may increase soon after administration of vitamin K antagonists due to the rapid reduction of factors with a lower half-life, especially FVII.

Coumarins (2*H*-chromene-2-one or 1-benzopyran-2-one) are a class of naturally occurring coumarins, which have been shown to possess other important pharmacological activities such as anti-inflammatory, antibacterial, antifungal, antiviral, anticancer, antihypertensive, antitubercular, anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant, and neuroprotective properties.[8] Considering the anticoagulant activity, 4-hydroxycoumarins represent the VKAs in therapy possessing a bulky substituent at the 3-position. Coumarins other than 4-hydroxycoumarins drugs with a bulky substituent at the 3- and 4-position, were also described with anticoagulant activity, namely a series of coumarins 3,4-cyclocondensed *O*-glycopyranosides.[9] A series of 6-substituted coumarins was also found to act as inhibitors of FXa[10] and some sulfated coumarins were already described as allosteric regulators of thrombin.[11]

In the last decade we have been working on the sulfation of several glycosidic polyphenolic small molecules, namely coumarins,[12] flavonoids,[13] xanthones[14] and found promising anticoagulant actions within these scaffolds. For instance, the persulfate derivative of the naturally-occurring coumarin, esculin (**Figure 2.1**), was able to prolong APTT and PT, APTT more than PT, with no influence on TT pathway,[12] and more recently, Ahmad *et al.* showed that our previously persulfated esculin, was also active *in vivo*, being able to decrease the thrombus formation when administered in occlusion induced thrombotic rats.[15] Therefore, the validation of this lead compound offers an opportunity to further investigate this scaffold, and other naturally occurring and synthetic coumarins in the pursuit of new anticoagulant agents.

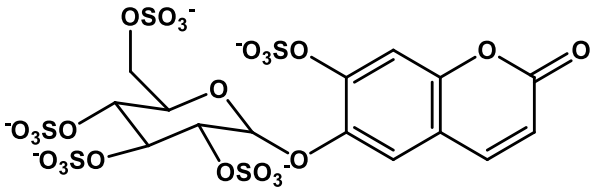


Figure 2.1. The chemical structure of persulfated esculin.

In this work, a triple hybridization approach was planned from naturally occurring and synthetic coumarins bearing sugar sulfated portion and a triazole moiety (**Figure 2.2**).

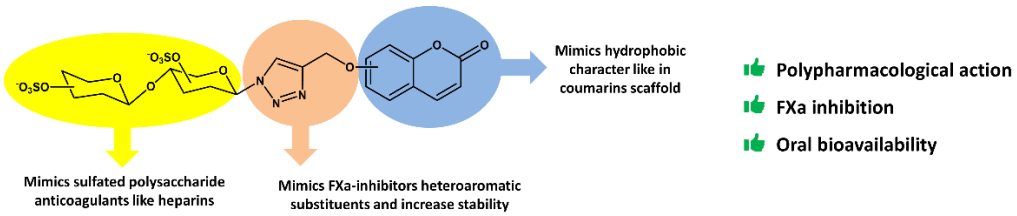


Figure 2.2. Strategy for new coumarins-based anticoagulants agents.

The general structure was planned to mimetize the sulfated polysaccharide anticoagulants (with the sugar sulfated moiety) such heparin, yet lacking the high anionic character, while adding some hydrophobic character (with the coumarinic scaffold). Furthermore, the triazole moiety was introduced as a linker between coumarin and the sugar sulfated moiety, taking in consideration the literature evidences that show heteroaromatic rings as having an important role in the inhibition of FXa.[16] Furthermore, adding a triazole linker could increase the metabolic stability of our previously obtained *O*-glycosidic persulfates. For structure-activity relationship purposes two different glycosidic

moieties (glucose or cellobiose) were selected in order to verify the influence of the sugar on the anticoagulant activity.

2.2. Results and discussion

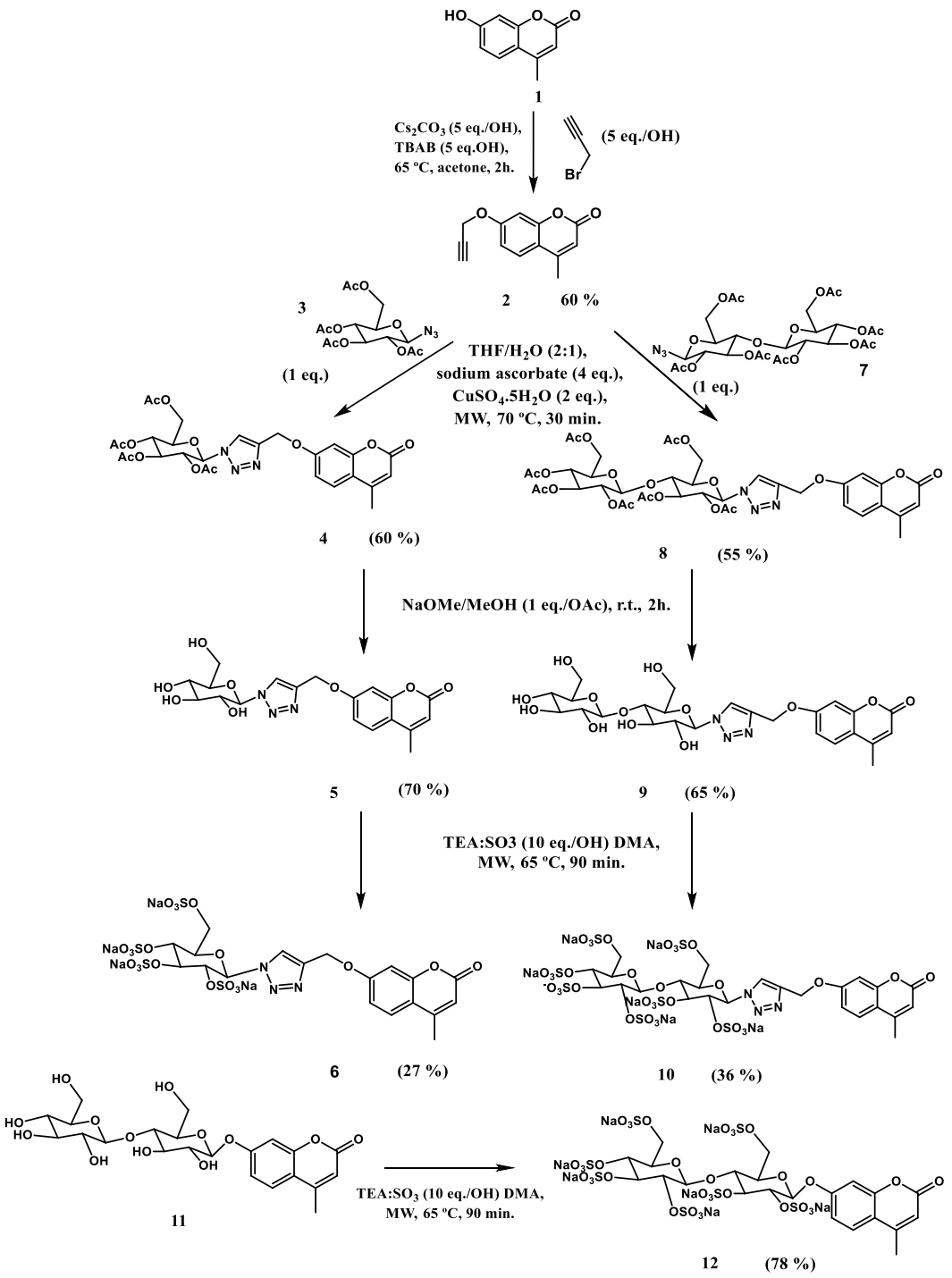
2.2.1. Synthesis of sulfated glycosyl coumarin hybrids

Click chemistry has been applied to produce several types coumarin-saccharides for other activities such as the fluorescent activity[17] and inhibitory activity of carbonic anhydrase IX and XII.[18] Particularly, the copper(I)-catalyzed azide-alkyne reaction (CuAAC) is a powerful strategy to generate the 1,2,3-triazole moiety, a bioisoster of N-heterocycle motifs present in FXa inhibitors.[19]

In this work, seven 7-substituted coumarins (**Scheme 2.1**; **2** – **12**) and four 6-substituted coumarins (**Scheme 2.2**; **14** – **17**) were synthesized.

In order to obtain the building blocks for the click chemistry reaction, 7-methylumbelliferone (**1**) and 6-hydroxy-2*H*-chromene-2-one (**13**) were selected for propargylation (**Scheme 2.1** and **2.2**). The reaction did not occur at room temperature (r.t.), but the reaction occurred smoothly in 1 hour at 65 ˚C in acetone. 4-Methyl-7-(prop-2-yn-yloxy)umbelliferone (**2**) and 6-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**14**) were obtained after crystallization. These two compounds were previously obtained[17] with $CHCl_3/H_2O$ as a solvent, and following by liquid-liquid extraction, according to the authors, in terms of equivalents, two equivalents of Na_2CO_3, one for TBAB, and one for propargyl bromide were used.

Coumarins **2** and **14** were subsequently linked to tetraacetate *β*-glucopyranosyl azide (**3**) and heptaacetate-*β*-D-cellobiosyl azide (**7**), by CuAAC in the presence of $CuSO_4.5H_2O$ and sodium ascorbate in mixture of tetrahydrofuran/water (2:1) for 30 minutes at 70 ˚C under microwave irradiation (MW) at 300W (**Scheme 2.1**). Microwave irradiation is a powerful assistant for enhancing the reactivity and tremendously economizing the reaction time.[20] 4-Methyl-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**4**), 4-methyl-(1-(1(-2,3,6,2',3',4',6'-hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**8**) and 6-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**15**) were obtained varying from 30 min to 1h. (**Scheme 2.1** and **2.2**). Flash column chromatography was necessary for the isolation of the click products. Coumarins **4** and **15** were previously obtained by CuAAC reaction,[17] however with phase transference catalysis (PTC) and conventional heating at room temperature.

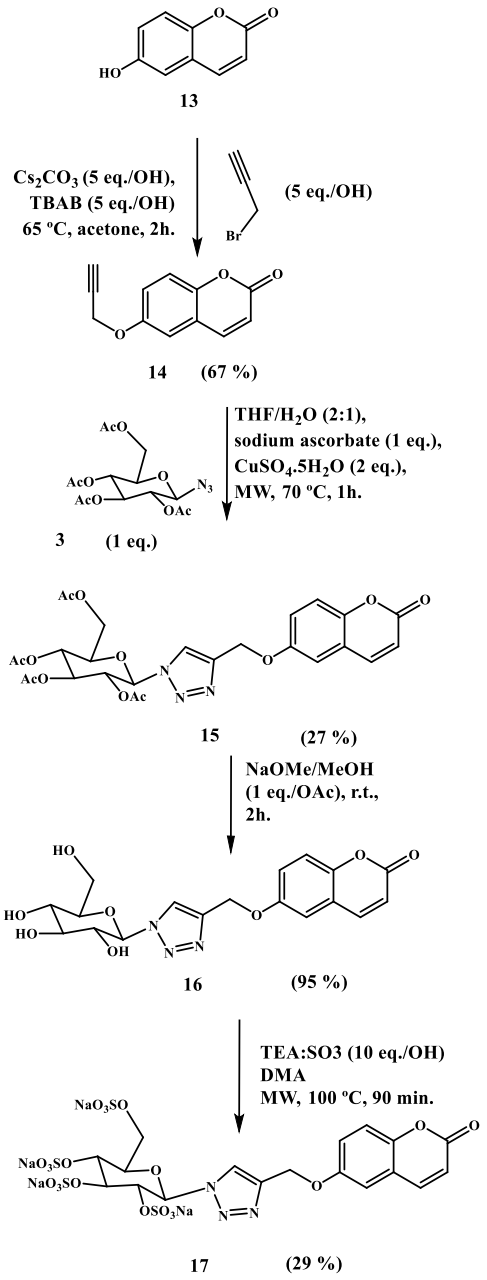


Scheme 2.1. Synthesis of 4-methyl-7-substituted coumarins.

Coumarins 4-methyl-(1-(1-(*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy) umbelliferone (**5**), 4-methyl-(1-(1(-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy) umbelliferone (**9**), and 6-(1-(1-(*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**16**) were obtained, after deprotection of the coumarins **4**, **8**, and **15** with sodium methoxide, overnight (**Scheme 2.1** and **2.2**).[21] Neutralization of the crude product was achieved by strong cationic exchange with solid phase extraction cartridges.

In order to obtain the persulfate derivative of coumarins **5**, **9**, and **16** the reactions were accomplished with 10 eq./OH of triethylamine sulfur trioxide (SO_3:TEA adduct) in DMA solution at 65 ˚C under MW irradiation 200 W, varying the reaction times between 90 min. to 120 min. (**Scheme 2.1** and **2.2**),[13] resulting for the first time, in synthesis sulfated glycosyl coumarins linked to triazoles: 4-methyl-(1-(1-(2,3,4,6-tetrasulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**6**), 4-methyl-(1-(1-(2,3,6,2',3',4',6'-heptasulfate-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4yl)methoxy)umbelliferone (**10**), 4-methyl-(1-(1-(2,3,6,2',3',4',6'-heptasulfate-*β*-D-cellobiosyl)methoxy)umbelliferone (**12**) and 6-(1-(1-(2,3,4,6-tetrasulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**17**). To remove water-soluble impurities, dialysis using a Spectra/Por6 regenerated cellulose membrane (MW1000) was applied.

To study the influence of the position of the triazole-linked sulfated saccharide on the coumarin scaffold 6-glycosyl triazole coumarins were also obtained (**Scheme 2.2**).



Scheme 2.2. Synthesis of 6-substituted coumarins.

The synthesized coumarins were fully characterized by infrared (IR), proton nuclear magnetic resonance (^{1}H NMR), 13 carbon nuclear magnetic resonance (^{13}C NMR) and heteronuclear single quantum coherence spectroscopy (HSQC)/heteronuclear multiple-bond correlation spectroscopy (HMBC) techniques. The IR spectra of propargylated coumarins **2** and **14** showed a C≡C-bond stretching at around 3304 - 3226 cm^{-1} which confirms the presence of the alkyne moiety to the coumarin core. The ^{1}H NMR spectra of coumarins **2** and **14** showed one doublet signal of two protons at δ_H 4.94 ppm (J=2.4 Hz) and δ_H 4.86 ppm (J=2.3 Hz), respectively, which was assigned to the methylene protons as

well as a characteristic singlet of an alkyne proton at δ_H 3.64 ppm. ^{13}C NMR spectra of coumarins **2** and **14** showed three signals between δ_C 78.9 - 56.0 ppm characteristic of the three alkylic carbons, which were not observed in the ^{13}C NMR specta of the unmodified building blocks **1** and **13**.

The IR spectra of acetylated coumarins **4**, **8**, and **15** showed a band at 1741 - 1749 cm^{-1} associated with C=O of an ester group. In the ^1H NMR of these coumarins (**4**, **8**, and **15**), two singlets at δ_H 8.60 - 8.57 ppm (1H, triazole *H*) and 5.30 - 5.27 ppm (2H, OCH_2) were observed confirming the presence of a triazole ring. The ^{13}C NMR spectra showed also signals at δ_C 142.8 - 142.7 ppm (*C*=CH triazole) and at δ_C 126.6 - 124.0 ppm (*C*H=C triazole), corresponding to the carbons of the triazole ring (**Figure 2.3**) and a signal at δ_C 61.5 ppm, corresponding to the methylenic protons of ether bond (OCH_2). In HMBC it was possible to confirm the triazole linkage to coumarin due to the correlation between the two protons at δ_H 5.30 - 5.27 ppm (OCH_2) and C6 or C7 (δ_C 60.5 – 60.0 ppm) (**Figure 2.3**). It was possible to confirm that the saccharidic moieties kept the *β* configuration due to the high coupling constant of H-1' (*J*= 9.0 Hz).[22]

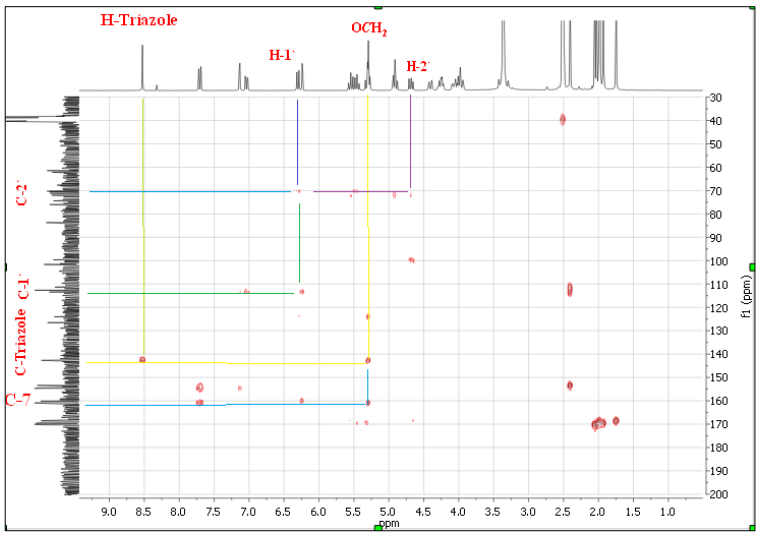


Figure 2.3. Heteronuclear multiple-bond correlation spectroscopy (DMSO-d_6) of the 7-cellobiosyl triazole coumarin **8**.

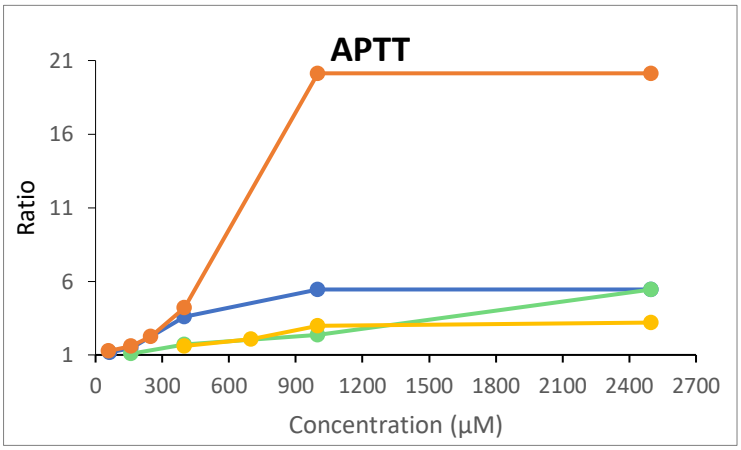
Hydroxylated glycosyl triazole coumarin **5**, **9**, and **16** showed an O-H-bond around 3442 - 3469 cm^{-1} associated with hydroxyl groups confirming the success of the deacetylation of these coumarins and the presence of a band at 1688 cm^{-1} typical of a carbonylic vibration of a carboxylic acid. In the ^1H NMR of these hydroxylated coumarins

signals between δ_H 2.05 and 1.72 ppm, or around δ_C 20 ppm were absence for both coumarins, allowed to confirm that the deacetylation was successfully achieved.

The IR spectra of sulfated coumarins **6**, **10**, **12**, and **17** showed the following important bands around 1260 cm^{-1} (S=O), 1030 cm^{-1} (C-O-S), and 800 cm^{-1} (S-O). Comparing ^{1}H NMR spectra of sulfated coumarins **6**, **10**, and **17** with the parent non-sulfated coumarins **5**, **9**, and **16**, respectively some signals of glycosidic have remained under the watermark; however, it was possible observe a slight deviation to higher values of chemical shift, confirming the presence of sulfate groups.

2.2.2. Anticoagulant activity

The anticoagulant activity of sulfated coumarins **6**, **10**, **12**, and **17** was measured in human plasma by the classical clotting times - activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) – in five different concentrations. The non-sulfated coumarins **5**, **9**, **11**, and **16** were evaluated at the highest concentration tested for the corresponding sulfated derivatives in order to explore the influence of the sulfate group on the anticoagulant activity. The results for the sulfated derivative **6**, **10**, **12**, and **17** on APTT, PT, and TT are summarized in **Figure 2.4** and the concentration needed to double the coagulation time ($APTT_2$, PT_2 or TT_2) were calculated in **Table 2.1**.



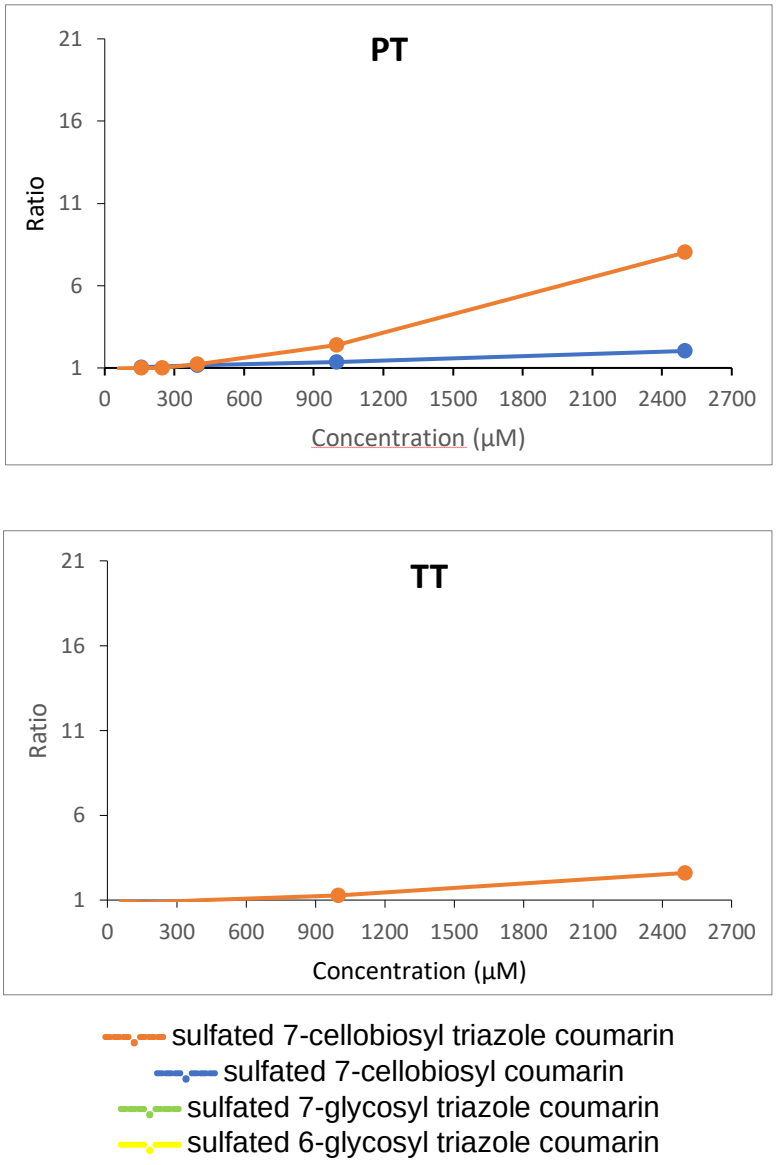


Figure 2.4. Dose-dependent effects of persulfated 6- and 7-glycosyl coumarins on APTT, PT and TT clotting assays using human pooled plasma, expressed as ratio of clotting time.

The tested sulfated coumarins **6**, **10**, **12**, and **17** showed anticoagulant properties in a dose dependent manner (**Figure 2.4**). The APTT was the most sensitive test to the presence of sulfated coumarins and these sulfated coumarins were able to completely block this clotting time at highest concentration (2500 µM) (**Figure 2.4**). Coumarins **6** and **17** were only able to impact the APTT ($APTT_2$ = ± 600 µM). Sulfated coumarin **10** and **12** were able to prolong both APTT and PT test. Only sulfated **10** was found to double the three clotting times (**Figure 2.4**). Non-sulfated coumarins (**5**, **9**, **11**, and **16**) were inactive even at the highest concentration tested for corresponding the sulfated derivatives (results not shown).

Table 2.1. Concentrations (μM) of persulfated 6- and 7-glycosyl coumarins required to double clotting assays.

	$APTT_2$	PT_2	TT_2
6	**669 ± 28**	**N.A.**	**N.A.**
10	**216 ± 77**	**812 ± 56**	**1863 ± 270**
12	**222 ± 1**	**2439 ± 174**	**N.A.**
17	**657 ± 15**	**N.A.**	**N.A.**

N.A. not active at 2500 μM.

The concentration needed to double each coagulation time was calculated (**Table 2.1**). The most promising sulfates were **10** and **12** with $APTT_2$ = ± 200 μM. This proves that coumarin-related "cellobiose" favors anticoagulant activity. Coumarins **10** and **12** contain higher number of sulfate groups than esculin persulfate (**Figure 2.1**), which exhibited an $APTT_2$ around 300 μM,[12] confirming our previous conclusions that pointed out that the anticoagulant activity increases with the increase number of sulfate groups.[13-14, 23]

Prolongation of APTT test indicates that an inhibition of the coagulation factors kininogen, prekallikrein, XII, XI, IX, VIII, X, V, I, and II, can be underlying the mode of action of coumarins **6**, **10**, **12**, and **17**. 7-Glycosyl coumarin **12** and 7-glycosyl triazole coumarin **10** prolonged the PT assay (PT_2 = ± 2000 and ± 800 μM, respectively) so the factors: VII, X, V, II, and I were possibly inhibited. Only 7-glycosyl triazole coumarin **10** prolonged APTT, PT and TT assay (TT_2 = ± 1800 μM), this coumarin affects the 3 stages of coagulation, then the factors that are common to both pathways may be inhibited. Coumarin **10** seems to be a potential inhibitor of FXa and so we will look further ahead. As FXa inhibition of its activity disrupts fibrin formation initiated in each of them, may, therefore, justify a superior anticoagulant effect.

2.3. Experimental section

2.3.1. Materials and methods

4-Methylumbelliferone (**1**) and potassium carbonate were purchased from Aldrich; TBAB, tetra-*n*-butylammonium bromide from TCI; propargyl bromide solution 80% wt. % in toluene from Acros Organics, sodium ascorbate, sodium methoxide, triethylamine sulfur trioxide adduct, 4-methyl-(1-(1-(*β*-D-cellobiosyl)methoxy)umbelliferone (**11**), 6-hydroxy-2*H*-chromene-2-one (**13**) and TBAHS, Sigma; copper(II) sulfate 5-hydrate from Panreac; *β*-D-cellobiosyl azide heptaacetate **7** from Synthose and cesium carbonate from Fluka. APTT, PT and TT tests were performed using a coagulometer machine SYSMEX CA-540, serial number: A2307, software 00-17. The following commercial kits were used: DADE® ACTIN® reagent ref. B4218-20, lot. 538542 and $CaCl_2$ ref. ORHO-37, lot. 563828 for the APTT, TROMBOREL® S reagent ref. OUHP-29, lot. 562851 for the PT and TEST THROMBIN reagent ref. OWHM-13, lot. 556832 (reagent diluent lot. 557303) for the TT test. MW reactions were performed in sealed reaction vessels (30 mL) in a MicroSYNTH 1600 synthesizer from Milestone (ThermoUnicam, Portugal). The internal reaction temperature was controlled by a fiber-optic probe sensor. All the reactions were monitored by thin-layer chromatography (TLC) perform on pre-coated plates of silica gel (60 F254 Merck). The visualization of the chromatograms was under UV light at 254 and 365 nm. Solvents were evaporated using rotary evaporator under reduced pressure (Buchi Waterchath B-480). Melting points were obtained in a Köfter microscope and are uncorrected. IR spectra were measured on an ATI Mattson Genesis series FTIR spectrophotometer (software WinFirst, version 2.10), in KBr microplates (cm^{-1}). Purifications were performed by flash column chromatography using Macherey-Nagel silica gel 60 (0.04-0.063 mm); solid phase extraction (SPE) discovery DSC-SCX and dialysis (Spectra/Por6 regenerated cellulose membrane MWCO1000). ^{1}H and ^{13}C NMR spectra were performed in the Department of Chemistry of the University of Aveiro and were taken in DMSO-d_6 or D_2O at room temperature on Bruker Avance 300 instruments (300.13 or 500.16 MHz for ^{1}H and 75.47 or 125.77 MHz for ^{13}C). Chemical shifts are expressed as δ (ppm) values relative to tetramethysilane (TMS) as an internal reference. Coupling constants (J) are reported in hertz (Hz). Assignment abbreviations are the following: singlet (s), doublet (d), triplet (t), multiplet (m) and doublet of doublets (dd).

2.3.2. Synthesis

4-Methyl-7-(prop-2-yn-yloxy)umbelliferone (**2**)

To a solution of the 4-methylumbelliferone (**1**) (500 mg, 2.84 mmol), potassium carbonate (4630 mg, 14.2 mmol, 5 eq./OH) and tetra-*n*-butylammonium bromide (4580 g, 14.2 mmol, 5 eq/OH) in acetone (40 mL), propargyl bromide solution 80% wt. % in toluene (3.00 mL, 14.2 mmol, 5 eq./OH) was added. The mixture was refluxed at 60 °C for 2 hours and filtered. After concentration under reduced pressure, the oil was purified by crystallization (methanol) to give a 4-methyl-7-(prop-2-yn-yloxy)umbelliferone (**2**) as a white solid (374 mg, 60 %). The chromatographic control was performed by TLC (silica gel, chloroform/methanol, 95:5).

M.p.: 161 – 163 °C. IR υmax (cm^{-1}) (KBr): 3304, 1765, 1722. ^{1}H-NMR (DMSO-d_6, 300.13 MHz) δ (ppm): 7.71 (1H, d, *J*=8.6 Hz, H-5), 7.03 (1H, dd, *J*=3.1 and 2.7 Hz, H-6), 7.00 (1H, d, *J*=2.6 Hz, H-8), 6.24 (1H, d, *J*=1.2 Hz, H-3), 4.94 (2H, d, *J*=2.4 Hz, OCH_2), 3.64 (1H, s, H-alkyne), 2.40 (3H, s, CH_3). ^{13}C-NMR (DMSO-d_6, 75.47 MHz) δ (ppm): 162.5 (C-7), 161.8 (C-2), 155.3 (C-8a), 155 (C-4), 127.6 (C-5), 114.3 (C-4a), 113.1 (C-6), 110.9 (C-3), 103.2 (C-8), 78.9 (C-alkyne), 78.6 (*C*H-alkyne), 56.0 (O*C*H_2), 20.0 (*C*H_3)

6-(Prop-2-yn-yloxy)-2*H*-chromene-2-one (**14**)

6-(Prop-2-yn-yloxy)-2*H*-chromene-2-one (**14**) was prepared from 6-hydroxy-2*H*-chromene-2-one (**13**) (500 mg, 3.08 mmol), like the preparation of coumarin 4-methyl-7-(prop-2-yn-yloxy)umbelliferone (**2**), to obtain the desired product as a yellow solid (412 mg, 67%). The chromatographic control was performed by TLC (silica gel, chloroform/methanol, 95:5).

M.p.: 162 - 164 °C. IR υmax (cm^{-1}) (KBr): 3226, 2360, 2342, 1707. ^{1}H-NMR (DMSO-d_6, 300.13 MHz) δ (ppm): 8.02 (1H, d, *J*=9.6 Hz, H4), 7.38 (1H, d, *J*=9.0 Hz, H-8), 7.34 (1H, d, *J*=2.9 Hz, H-5), 7.26 (1H, dd, *J*=9.0 and 2.9 Hz, H-7), 6.50 (1H, d, *J*=9.6 Hz, H-3), 4.86 (2H, d, *J*=2.3 Hz, OCH_2), 3.61 (1H, s, H-alkyne). ^{13}C-RMN (DMSO-d6, 75.47 MHz) δ (ppm): 160.1 (C-2), 153.4 (C-6), 148.2 (C-8a), 144.0 (C-4), 120.0 (C-4a), 119.2 (C-7), 117.4 (C-8), 116.8 (C-3), 112.3 (C-5), 78,9 (*C*H-alkyne), 78.6 (*C*H-alkyne), 56.0 (O*C*H_2).

4-Methyl-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**4**)

To a solution of 4-methyl-7-(prop-2-yn-yloxy)umbelliferone (**2**) (250 mg, 1.4 mmol) and 2,3,4,6-tetra-*O*-acetyl-*β*-glucopyranosyl azide (**3**) (520 mg, 1.4 mmol, 1 eq) in tetrahydrofuran/water (2:1, 50 mL), sodium ascorbate (1110 mg, 5.6 mmol, 4 eq.) and copper(II) sulfate 5-hydrate (700 mg, 2.8 mmol 2 eq.) were added. The reaction vessel was sealed, and the mixture was kept stirring and heated for 30 minutes at 70 °C under MW irradiation of 500 W (2 cycles: 5 minutes to achieve 70 °C and 10 minutes under 70 °C). After cooling, the reaction mixture was filtered. After concentration under reduced pressure, the water suspension was extracted with ethyl acetate (2 x 20 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered, concentrated under reduced pressure, and then purified by solid phase extraction cartridge (petroleum ether/ethyl acetate, 5:5) to obtain 4-methyl-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**4**) as a white solid (497 mg, 60 %). The chromatographic control was performed by TLC (silica gel, chloroform/methanol, 80:20).

M.p.: 194 - 197 °C. IR umax (cm^{-1}) (KBr): 1742, 1620, 1429. ^{1}H-NMR (DMSO-d_6, 300.13 MHz) δ (ppm): 8.60 (1H, s, H-triazole), 7.70 (1H, d, *J*=8.6 Hz, H-5), 7.13 (1H, d, *J*=2.5 Hz, H-8), 7.04 (1H, dd, *J*=2.5 and 2.5 Hz, H-6), 6.38 (1H, d, *J*=9.1 Hz, H-3), 6.23 (1H, d, *J*=1.2 Hz, H-1'), 5.67 (1H, t, *J*=9.3 Hz, H-2'), 5.56 (1H, t, *J*=9.4 Hz, H-3'), 5.30 (2H, s, OC*H*$_2$), 5.18 (1H, t, *J*=9.7 Hz, H-4'), 4.40 - 4.34 (1H, m, H-5'), 4.17 - 4.05 (2H, m, H-6'a, H-6'b), 2.40 (3H, s, C*H*$_3$), 2.03 (3H, s, COOC*H*$_3$), 2.00 (3H, s, COOC*H*$_3$), 1.97 (3H, s, COOC*H*$_3$), 1.76 (3H, s, COOC*H*$_3$). ^{13}C-RMN (DMSO-d_6, 75.47 MHz) δ (ppm): 170.1 (1 *C*OOCH3), 169.6 (1 *C*OOCH3), 169.4 (1 *C*OOCH3), 168.5 (1 *C*OOCH3), 160.9 (C-2), 160.2 (C-6), 154.7 (C-8a), 153.5 (C-4), 142.8 (*C*=CH triazole), 126.6 (*C*H=C triazole) 124.0 (C-7), 113.5 (C-4a), 112.7 (C-8), 111.4 (C-3), 101.7 (C-5), 83,9 (C-1'), 73.3 (C-5'), 72.2 (C-3'), 70.1 (C-2'), 67.5 (C-4'), 61.8 (C-6'), 61.5 (O*C*H$_2$), 20.5 (COO*C*H3), 20.4 (COO*C*H3), 20.3 (COO*C*H3), 19.9 (COO*C*H3), 18.1 (*C*H$_3$).

4-Methyl-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**8**)

4-Methyl-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**8**) was prepared from 4-methyl-7-(prop-2-yn-yloxy)umbelliferone (**2**) (200 mg, 1.13 mmol) and *β*-D-cellobiosyl azide heptaacetate (**7**) (751 mg, 1.13 mmol, 1 eq.) in the same conditions from reaction 4-methyl-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-

glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**4**) After concentration under reduced pressure, the water suspension was extracted with chloroform (2 x 30 mL). The purification was by crystallization (methanol) to give 4-methyl-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**8**) as white solid (508 mg, 54 %). The chromatographic control was performed by TLC (silica gel, chloroform/acetone, 85:15).

M.p.: 198 - 199 °C. IR umax (cm^{-1}) (KBr): 3470; 3160; 2963; 1746; 1617. ^{1}H-NMR (DMSO-d_6,300.13 MHz) δ (ppm): 8.51 (1H, s, H-triazole), 7.68 (1H, d, *J*=8.8 Hz, H-5), 7.11 (1H, d, *J*=2.4 Hz, H-8), 7.02 (1H, dd, *J*=2.5 and *J*=2.5 Hz, H-6), 6.28 (1H, d, *J*=9.0 Hz, H-1`), 6.21 (1H, d, *J*=1.1 Hz, H-3), 5.55 – 5.40 (2H, m, H-cellobiose), 5.31 – 5.25 (1H, m, H-cellobiose), 5.27 (2H, s, $OCH_2$), 4.92 – 4.86 (2H, m, H-cellobiose), 4.68 (1H, dd, *J*= 9.6 and 8.1 Hz, H-2`), 4.38 (1H, d, *J*=11.2 Hz, H-cellobiose), 4.27 – 4.19 (2H, m, H-cellobiose), 4.08 – 3.90 (5H, m, H-cellobiose), 2.38 (3H, s, C*H*$_3$), 2.03 – 1.91 (18H, m, COOC*H*$_3$), 1.73 (3H, s, COOC*H*$_3$). ^{13}C-NMR (DMSO-d_6, 75.47 MHz) δ (ppm):170.3 (1 *C*OOCH$_3$), 170.1 (1 *C*OOCH$_3$), 169.6 (1 *C*OOCH$_3$), 169.5 (1 *C*OOCH$_3$), 169.2 (1 *C*OOCH$_3$), 169.0 (1 *C*OOCH$_3$), 168.5 (1 *C*OOCH$_3$), 160.8 (C-2), 160.2 (C-7), 154.6 (C-8a), 153.4 (C-4), 142.7 (*C*=CH triazole), 126.5 (C-5), 124.0 (*C*H=C triazole), 113.4 (C-4a), 112.7 (C-6), 111.3 (C-3), 101.6 (C-8), 99.6 (C-7`), 83.7 (C-1`), 76.0 (C-4`), 74.4 (C-5`), 72.2 (C-cellobiose), 72.0 (C-cellobiose), 71.8 (C-cellobiose), 70.5 (C-cellobiose), 70.3 (C-cellobiose), 62.2 (C-cellobiose), 61.5 (C-cellobiose), 61.5 (O*C*H$_2$), 20.6 (COO*C*H$_3$), 20.4 (COO*C*H$_3$), 20.3 (COO*C*H$_3$), 20.2 (COO*C*H$_3$), 20.1 (COO*C*H$_3$), 19.8 (COO*C*H$_3$), 18.1 (*C*H$_3$).

6-(1-(1-(2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4 yl)methoxy)-2*H*-chromene-2-one (**15**)

6-(1-(1-(2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**15**) was prepared from 6-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**14**) (400 mg, 2 mmol) and tetraacetate *β*-glucopyranosyl azide (**3**) (750 g, 2 mmol, 1 eq.) in the same conditions than coumarin 4-methyl-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**4**). The purification was by solid phase extraction cartridge (petroleum ether/ethyl acetate, 5:5) to obtain 6-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy) -2*H*-chromene-2-one (**15**) as a white solid (309 mg, 27 %). The chromatographic control was performed by TLC (silica gel, petroleum ether/ethyl acetate 5:5).

M.p.: 210 – 212.5 °C. IR υmax (cm^{-1}) (KBr): 1749, 1630, 1492. ^{1}H-NMR (DMSO-d_6, 300.13 MHz) δ (ppm): 8.57 (1H, s, H-triazole), 8.00 (1H, d, *J*=5.7 Hz, H-4), 7.40 (1H, d, *J*=1.8 Hz, H-5), 7.36 (1H, d, *J*=5.4 Hz, H-8), 7.28 (1H, dd, *J*=5.4 and 1.8 Hz, H-7), 6.51 (1H, d, *J*=5.7 Hz, H-3), 6.38 (1H, d, *J*=5.5 Hz, H-1'), 5.67 (1H, t, *J*=5.6 Hz, H-2'), 5.56 (1H, t, *J*=5.7 Hz, H-3'), 5.23 (2H, s, OC*H*$_2$), 5.20 - 5.16 (1H, m, H-4'), 4.39-4.35 (1H, m, H-5'), 4.15 - 4.06 (2H, m, H-6'a, H-6'b) 2.03 (3H, s, COOC*H*$_3$), 2.00 (3H, s, COOC*H*$_3$), 1.97 (3H, s, COOC*H*$_3$), 1.76 (3H, s, COOC*H*$_3$). ^{13}C-RMN (DMSO-d_6, 75.47 MHz) δ (ppm): 170.1 (1 *C*OOCH3), 169.6 (1 *C*OOCH$_3$), 169.4 (1 *C*OOCH$_3$), 168.5 (1 *C*OOCH$_3$), 160.1 (C-2), 154.1 (C-6), 148.1 (C-8a), 144.1 (C-4), 143.2 (*C*=CH triazole), 123.7 (*C*H=C triazole) 120.1 (C-7), 119.2 (C-4a), 117.4 (C-8), 116.7 (C-3), 112.1 (C-5), 83,8 (C-1'), 73.2 (C-5'), 72.1 (C-3'), 70.1 (C-2'), 67.5 (C-4'), 61.8 (C-6'), 61.5 (O*C*H2), 20.5 (COO*C*H$_3$), 20.4 (COO*C*H$_3$), 20.3 (COO*C*H$_3$), 19.9 (COO*C*H$_3$).

4-Methyl-(1-(1-(*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**5**)

To a solution of 4-methyl-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**4**) (300 mg, 0.51 mmol) in anhydrous methanol (20 mL), 0.5 M sodium methoxide (1.1 mL, 0.51 mmol, 1 eq./OAc) was added and the reaction mixture was stirred at room temperature overnight. The reaction mixture was filtered and a white solid was insolubilized from the reaction mixture 4-methyl-(1-(1-(-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**5**) was obtained as a pure solid (148 mg, 70 %). The chromatographic control was performed by TLC (silica gel, chloroform/methanol 90:10).

M.p.: 220 – 223 °C. IR υmax (cm^{-1}) (KBr): 3443, 1689, 1449. ^{1}H-NMR (DMSO-d_6, 300.13 MHz) δ (ppm): 8.50 (1H, s, H-triazole), 7.71 (1H, d, *J*=8.9 Hz, H-5), 7.17 (1H, d, *J*=2.5 Hz, H-8), 7.06 (1H, dd, *J*=2.5 and 2.5 Hz, H-6), 6.23 (1H, d, *J*=1.2 Hz, H-3), 5.57 (1H, d, *J*=9.3 Hz, H-1'), 5.44 – 5.20 (5H, m, H.2', H-3', OCH$_2$, H-4'), 4.66 (1H, s, H-5'), 3.81 – 3.68 (2H, m, H-6'a, H-6'b), 3.26 – 3.16 (2H, m, OH), 2.52 – 2.40 (2H, m, OH). ^{13}C-NMR(DMSO-d_6, 75.47 MHz) δ (ppm): 161.1 (C-2), 160.2 (C-6), 154.7 (C-8a), 153.5 (C-4), 142.0 (*C*=CH triazole), 126.6 (*C*H=C triazole) 124.3 (C-7), 113.5 (C-4a), 112.6 (C-8), 111.4 (C-3), 101.6 (C-5), 87.6 (C-1'), 80.0 (C-5'), 77.0 (C-3'), 72.1 (C-2'), 69.6 (C-4'), 61.6 (C-6'), 60.8 (O*C*H$_2$).

4-Methyl-(1-(1(-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**9**)

4-Methyl-(1-(1(-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**9**) was prepared from 4-methyl-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**8**) (200 mg, 2.23 mmol) in anhydrous methanol (25 mL) and 0.5 M sodium methoxide (3.20 mL, 7 eq./OH) and the reaction mixture was kept stirring at room temperature over 2 hours. The formed precipitate was filtered. The solid and filtered was purified by solid phase extraction (methanol) to obtain 4-methyl-(1-(1(-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**9**) as a white solid (128 mg, 65 %). The chromatographic control was performed by TLC (silica gel, chloroform/methanol 70:30).

M.p.: 222 – 224 °C. IR umax (cm^{-1}) (KBr): 3431, 2920, 2360, 1711, 1616. ^{1}H-NMR (DMSO-d_6, D_2O,300.13 MHz) δ (ppm): 8.32 (1H, s, H-triazole), 7.63 (1H, d, H-5), 6.98 (2H, d, *J*=7.4 Hz, H-8 and H-5), 6.13 (1H, s, H-3), 5.60 (1H, d, *J*=9.2 Hz, H-1`), 5.21 (2H, s, OCH_2), 4.33 (1H, d, *J*=7.7, H-1``), 3.38 – 3.07 (11H, m, H-cellobiose), 2.31 (3H, s, $CH_3$). $^{13}$C-NMR (DMSO-$d_6$, $D_2O$, 75.47 MHz) δ (ppm): 161.9 (C-2), 161.5 (C-7), 155.1 (C-4a), 142.8 (*C*=CH triazole), 127.3 (C-5), 124.9 (*C*H=C triazole), 114.2 (C-8a), 113.5 (C-6), 111.7 (C-3), 103.4 (C-1``), 102.1 (C-8), 87.7 (C-4`), 78.2 (C-cellobiose), 77.0 (C-cellobiose), 76.5 (C-cellobiose), 75.4 (C-cellobiose), 73.7 (C-cellobiose), 72.2 (C-cellobiose), 70.3 (C-cellobiose), 61.8 (O*C*H_2), 60.1 (C-cellobiose), 18.7 (*C*H_3).

6-(1-(1-(*β*-D-Glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**16**)

6-(1-(1-(*β*-D-Glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**16**) was prepared from 6-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**15**) (300 mg, 0.52 mmol) in the same conditions than coumarin 4-methyl-(1-(1(-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**9**) to obtain the desired product as an orange solid (199 mg, 95 %). The chromatographic control was performed by TLC (silica gel, chloroform/methanol 80:20).

M.p.: 231 – 233 °C. IR umax (cm^{-1}) (KBr): 3370, 1688, 1464. ^{1}H-NMR (DMSO-d6, 300.13 MHz) δ (ppm): 8.46 (1H, s, H-triazole), 8.02 (1H, d, *J*=9.5 Hz, H-4), 7.44 (1H, d, *J*=2.8 Hz, H-5), 7.37 (1H, d, *J*=9.0 Hz, H-8), 7.30 (1H, dd, *J*=2.9 and 2.8 Hz, H-7), 6.50 (1H, d, *J*=9.5 Hz, H-3), 5.56 (1H, d, *J*=9.3 Hz, H-1'), 5.40 – 5.15 (6H, m, OCH_2, OH), 4.61 (2H, s, H-2', H3'), 3.81 – 3.69 (4H, m, H-4', H-5', H-6'a, H-6'b). ^{13}C-NMR (DMSO-d_6, 75.47 MHz)

δ: 160.1 (C-2), 154.3 (C-6), 148.0 (C-8a), 144.0 (C-4), 142.2 (*C*=CH triazole), 124.0 (*C*H=C triazole) 120.0 (C-7), 119.2 (C-4a), 117.4 (C-8), 116.6 (C-3), 111.8 (C-5), 87.5 (C-1'), 79.9 (C-5'), 79.1 (C-3'), 76.9 (C-2'), 72.1 (C-4'), 69.6 (C-6'), 61.6 (O*C*H_2).

4-Methyl-(1-(1-(2,3,4,6-tetra-*O*-sulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl) methoxy)umbelliferone (**6)**

To a solution of 4-methyl-(1-(1-(-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl) methoxy)umbelliferone (**5**) (100 mg, 0.24 mmol) in DMA (10 mL), triethylamine sulfur trioxide adduct (1.7 g, 10 eq./OH) was added. The reaction vessel was sealed, and the mixture was kept stirring and heated for 90 minutes at 65 °C under microwave irradiation of 200W (3 cycles: 1 minute to achieve 100 °C and 30 minutes under 65 °C). After cooling, the mixture was poured into acetone (100 mL) under basic conditions (a few milliliters of triethylamine) and left at 4˚C overnight. The crude oil formed was washed with acetone and then dissolved in aqueous solution of 30 % sodium acetate (400 µL, 1 eq/SO_3^-). The suspension was added dropwise in ethanol to precipitate the sodium salt of the sulfated product. The solid obtained was further purified from other salts (monitored by IR) using a Spectra/Por 6 regenerated cellulose MWCO 1000 to obtain 4-methyl-(1-(1-(2,3,4,6-tetra-*O*-sulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**6**) as a brown solid (54 mg, 27%). The chromatographic control was performed by TLC (silica gel, ethyl acetate/methanol 80:20).

M.p.: 201 - 206 °C. IR υmax (cm^{-1}) (KBr): 1262, 1038, 800. ^{1}H-NMR (DMSO-d_6, 300.13 MHz) δ (ppm): 8.52 (1H, s, H-triazole), 7.71 (1H, d, *J*=8.8 Hz, H-5), 7.17 (1H, d, *J*=2.4 Hz, H-8), 7.12 (1H, dd, *J*=2.4 and 2.5 Hz, H-6), 6.22 (1H, t, *J*=2.6 Hz H-3), 5.22 (2H, s, OCH_2), 4.97 (1H, s, H-1'), 4.78 (1H, d, *J*=1.6 Hz, H-2'), 4.25 (1H, d, *J*=6.7 Hz, H-3'), 4.07 (1H, d, *J*=9.5 Hz H-4'), 3.99 – 3.93 (1H, m, H-5'), 3.67 – 3.61 (2H, m, H-6'a, H-6'b). ^{13}C-NMR (DMSO-d_6, 75.47 MHz) δ (ppm): 161.2 (C-2), 160.3 (C-6), 154.7 (C-8a), 153.6 (C-4), 141.9 (*C*=CH triazole), 126.6 (*C*H=C triazole) 124.4 (C-7), 113.4 (C-4a), 112.5 (C-8), 111.3 (C-3), 101.7 (C-5), 87.3 (C-1'), 75.8 (C-5'), 74.8 (C-3'), 73.6 (C-2'), 71.8 (C-4'), 66.6 (C-6'), 61.8 (O*C*H_2).

4-Methyl-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-sulfate-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**10**)

4-Methyl-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-sulfate-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**10**) was prepared from 4-methyl-(1-(1(-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**9**) (150 mg, 2.58 mmol) in the same conditions

than coumarin 4-methyl-(1-(1-(2,3,4,6-tetra-*O*-sulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**6**) to obtain a desired product as a brown solid (122 mg, 36 %). The chromatographic control was performed by TLC (silica gel, chloroform/methanol 40:60).

M.p.: 213 – 215 °C. IR umax (cm^{-1}) (KBr): 3462, 235 9, 1710, 1621. ^{1}H-NMR (DMSO-d_6, D_2O, 300.13 MHz) δ (ppm): 8.26 (H-triazole), 7.70 (1H, d, *J*=8.97 Hz, H-5), 7.05 (2H, s, H-8 and H-6), 6.30 (1H, d, *J*=6.31 Hz, H-1`), 6.15 (1H, s, H-3), 5.21 (2H, s, OC*H*$_2$), 5.09 (1H, d, *J*=5.10 Hz, H-2`), 4.86 (1H, m, H-cellobiose), 4.59 – 3.94 (under water, 10H, m, H-cellobiose), 2.35 (3H, s, C*H*$_3$). ^{13}C-NMR (DMSO-d_6, D_2O, 75.47 MHz) δ (ppm): 163.3 (C-2), 162.2 (C-7), 165.2 (C-8a), 155.5 (C-4), 142,8 (C=CH), 127.9 (C-5), 114.8 (CH=C), 114.2 (C-3), 112.1 (C-4), 102.7 (C-6), 100.4 (C-cellobiose), 78.8 (C-cellobiose), 78.5 (C-cellobiose), 78.4 (C-cellobiose), 76.8 (C-cellobiose), 74.3 (C-cellobiose), 72.2 (C-cellobiose), 68.7 (C-cellobiose), 62.4 (O*C*H$_2$), 19.2 (*C*H$_3$).

4-Methyl-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-sulfate-*β*-D-cellobiosyl)methoxy) umbelliferone (**12**)

4-Methyl-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-sulfate-*β*-D-cellobiosyl)methoxy) umbelliferone (**12**) was prepared from 4-methyl-(1-(1-(*β*-D-cellobiosyl)methoxy)umbelliferone (**11**) (400 mg, 0.80 mmol) in the same conditions than coumarin 4-methyl-(1-(1-(2,3,4,6-tetra-*O*-sulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**6**) obtaining a desired product an orange solid (758 mg, 78 %). The chromatographic control was performed by TLC (silica gel, methanol 100%).

M.p.: 220 - 223 °C. IR umax (cm^{-1}) (KBr): 1260, 1026, 804. ^{1}H-NMR (DMSO-d_6, 300.13 MHz) δ (ppm): 7.66 (1H, d, *J*=8.9 Hz, H-5), 7.14 (1H, dd, *J*=2.3 and 2.3 Hz, H-8), 7.02 (1H, d, *J*=2.3 Hz, H-6), 6.18 (1H, s, H-3), 5.72 (1H, d, *J*=5.4 Hz, H-1'), 4.71 (1H, d, *J*=3.2 Hz, H-1''), 4.61 (2H, t, *J*=5.1 Hz, H-2', H-2''), 4.40 (2H, d, *J*=4.2 Hz, H-3', H-3''), 4.27 (1H, d, *J*=6.0 Hz H-4''), 4.11 – 3.89 (7H, m, H-4', H-5', H-5'', H-6a', H-6a'', H-6b', H-6b''), 2.40 (7H, s, C*H*$_3$). ^{13}C-NMR (DMSO-d_6, 75.47 MHz) δ (ppm): 163.0 (C-2), 163.0 (C-7), 155,9 (C-8a), 155.0 (C-4), 127.8 (C-5), 115.6 (C-3), 114.7 (C-4a), 112.4 (C-6), 104.9 (C-8), 101.0 (C-1``), 97.3 (C-2``), 77.9 (C-3´), 77.7 (C-cellobiose), 77.3 (C-cellobiose), 76.7 (C-cellobiose), 76.6 (C-cellobiose), 76.5 (C-cellobiose), 74.1 (C-cellobiose), 72.3 (C-cellobiose), 68.8 (C-cellobiose), 68.5 (O*C*H$_2$), 19.2 (*C*H$_3$).

6-(1-(1-(2,3,4,6-Tetra-*O*-sulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl) methoxy)-2*H*-chromene-2-one (**17**)

6-(1-(1-(2,3,4,6-Tetra-*O*-sulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl) methoxy)-2*H*-chromene-2-one (**17**) was prepared from 6-(1-(1-(*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**16**) (200 mg, 0.49 mmol) in the same conditions than coumarin 4-methyl-(1-(1-(2,3,4,6-tetra-*O*-sulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**6**) obtaining a desired product as a yellow solid (117 mg, 29 %). The chromatographic control was performed by TLC (silica gel, ethyl acetate/methanol 80:20).

M.p.: 172 – 174 °C. IR umax (cm^{-1}) (KBr): 1261, 1036, 803. ^{1}H-NMR (DMSO-d_6, 300.13 MHz) δ (ppm): 8.54 (1H, s, H-triazole), 8.05 (1H, d, *J*=5.8 Hz, H-4), 7.46 (1H, d, *J*=1.1 Hz, H-5), 7.36 (1H, d, *J*=5.5 Hz, H-8), 7.32 (1H, dd, *J*=4.0 and 1.5 Hz, H-7), 6.48 (1H, d, *J*=5.7 Hz, H-3), 6.22 (1H, d, *J*=2.0 Hz, H-1'), 5.16 (2H, s, OC*H*$_2$), 4.98 (1H, s, H-2'), 4.25 (1H, d, *J*=4.1 Hz, H-3'), 4.07 (1H, t, *J*=3.2 Hz, H-4'), 3.97 – 3.94 (1H, m, H-5'), 3.66 – 3.62 (2H, m, H-6'a, H-6'b). ^{13}C-RMN (DMSO-d_6, 75.47 MHz) δ (ppm): 160.2 (C-2), 154.5 (C-6), 148.0 (C-8a), 144.3 (C-4), 142.2 (*C*=CH triazole), 124.3 (*C*H=C triazole) 120.4 (C-7), 119.2 (C-4a), 117.4 (C-8), 116.4 (C-3), 111.7 (C-5), 87.3 (C-1'), 75.7 (C-5'), 74.8 (C-3'), 73.5 (C-2'), 71.7 (C-4'), 66.5 (C-6'), 61.7 (O*C*H$_2$).

2.3.3. Biological activity

Human blood was collected from 5 healthy donors aged between 22 and 28 years old without history of bleeding or thrombosis and who had not taken any medication known to interfere with blood coagulation and platelet function for 2 weeks. Nine volumes of blood were decalcified with one volume of 3.8 % sodium citrate solution. Blood was centrifuged for 20 minutes at 2400 g and the pooled plasma was stored in aliquots at -20 ˚C until use.

Sulfated coumarins **6**, **10**, **12**, and **17** were dissolved in saline solution. Five dilutions were prepared for coumarins **6**, **10**, **12**, and **17** from 2500 to 60 µM. The non-sulfated coumarins **5**, **9**, **11**, and **16** were dissolved in saline solution with 10 % DMSO (2500 µM). In the control group, saline (control for sulfated coumarin) or saline solution with 10 % DMSO (control for non-sulfated coumarins) was used. Plasma and solution of the compounds were mixed in a 1:1 ratio.

The APTT reagent used was the synthetic phospholipids of purified soybean in ellagic acid 1×10^{-4} M, buffered, stabilized and preserved and calcium chloride (0.025 mol/L).

The PT reagent used was the lyophilized thromboplastin from human placenta (≤ 60 g/L), calcium chloride (~1.5 g/L) and stabilizers. The TT reagent used was lyophilized (1.5 UI /mL) bovine thrombin and bovine albumin.

The clotting times were recorded in seconds (s). Each measurement was performed in duplicate and repeated three times on different days (n=6). Coagulation time prolonging ratio was calculated comparing the clotting time in the presence of each concentration of tested compound with that obtained when saline was used instead of test compound. The concentration required to double the clotting time was calculated from linear regression analysis of each individual concentration-response curve. For clotting time values greater than 130 s (PT), 190 s (TT), and 600 s (APTT), values of 130 and 600 s, respectively, were arbitrarily assigned for statistical analysis.

Activated partial thromboplastin time (APTT)

The assay was carried out according to the respective instructions of the manufacture: citrated normal human plasma mixed (1:1) with sample solution (50 µL) was incubated for 1 min at 37 ˚C. Then APTT assay reagent (50 µL) was added and the mixture was incubated for 3 min at 37 ˚C. $CaCl_2$ (50 µL, 25 mmol/L) was added, and clotting times were recorded during 600 s.

Prothrombin time (PT)

The assay was carried out according to the respective instructions of the manufacture: citrated normal human plasma mixed (1:1) with sample solution (50 µL) was incubated for 3 min at 37 ˚C. Then PT assay reagent (100 µL), preincubated for 2 min at 37˚C, was added and clotting times were recorded during 130 s.

Thrombin time (TT)

The assay was carried out according to the respective instructions of the manufacture: citrated normal human plasma mixed (1:1) with sample solution (50 µL) was incubated for 2 min at 37 ˚C. Then 100 µL of thrombin solution, preincubated for 5 min at 37 ˚C, was added and clotting times were recorded during 190 s. The TT reagent used was 1.5 UI/mL of bovine thrombin and bovine albumin in five milliliters of buffer solution for test thrombin reagent HEPES (25 mmol/L), pH=7.4.

2.4. Conclusion

In this work, twelve coumarins derivatives **2**, **4** – **6**, **8** – **10**, **12**, **14** – **17** were synthesized from which four sulfated coumarins were synthesized for the first time. Anticoagulant activity was evaluated for coumarins **6**, **10**, **12**, and **17** in human pooled plasma. The tested sulfated coumarins **6**, **10**, **12**, and **17** showed anticoagulant properties in a dose dependent manner. The APTT assay was the most sensitive test to the presence of sulfated, inducing a mechanism of action similar to that of heparin. Coumarin **6** and **17** only able to impact the APTT ($APTT_2$ = ± 600 μM). Sulfated coumarin **10** and **12** was able to prolong both APTT and PT test. Only sulfated **10** was found to double three clotting times. The most promising sulfates were **10** and **12** with $APTT_2$ = ± 200 μM. Glycosyl-linked coumarins (**6**, **17** and our previously obtained esculin persulfate) were less potent than cellobiosyl coumarins (**10** and **12**), which evidences the influence of the presence of a disaccharide on anticoagulation activity. Triazole link seems to no effect anticoagulant activity but would favor the chemical stability of compounds.

Author Contributions:

Compounds **2** and **8** – **10** were synthesized by Ricardo Cristelo. Compounds **2** – **6** and **14** – **17** were synthesized by Catarina Carvalho. MCS and ES supervised the synthesis. The evaluation of anticoagulant activity was performed by Ricardo Cristelo, Catarina Carvalho, and Barbara Duarte. MCS, ES, and MP designed the study. FM supervised the anticoagulant assays. The manuscript was written by RC under the supervision of MCS and ES.

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Chapter 3 - Synthesis and structural elucidation of other small molecules containing saccharides

3. Synthesis and structural elucidation of other small molecules containing saccharides

Substituted coumarin containing cellobiose in position 6 and a glucose in position 4 were also planned (**Scheme 3.1** and **3.3**). The purpose of these syntheses was to investigate these compounds in future studies for their anticoagulant activity as well as the possible sulfated derivatives that could be obtained thereof.

3.1. Results and discussion

3.1.1. Synthesis

3.1.1.1. Synthesis of 6-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**18**)

Click chemistry is a term that was first proposed by Sharpless *et al.* in 2001; according to them, the click reactions must be modular, wide in scope, with very high yields, generate only inoffensive by-products and be stereospecific (but not necessarily enantioselective).[1] The traditional process of drug discovery based on natural secondary metabolites has often been slow, costly, and labour intensive. The required process includes simple reaction conditions (ideally, the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed, and simple product isolation. Purification, if required, must be by no chromatographic methods, such as crystallization or distillation, and the product must be stable under physiological conditions. The copper(I)-catalyzed 1,2,3-triazole formation from azides and terminal acetylenes (**Figure 3.1**) is a powerful tool for the generation of privileged medicinal scaffolds, due to its high degree of dependability, complete specificity, and the biocompatibility of the reactants.[2] Many researchers used click chemistry as a synthetic tool for the generation of pharmalogically valuable drugs.[3]

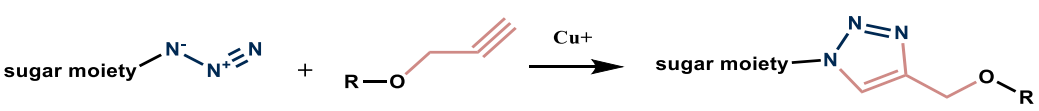
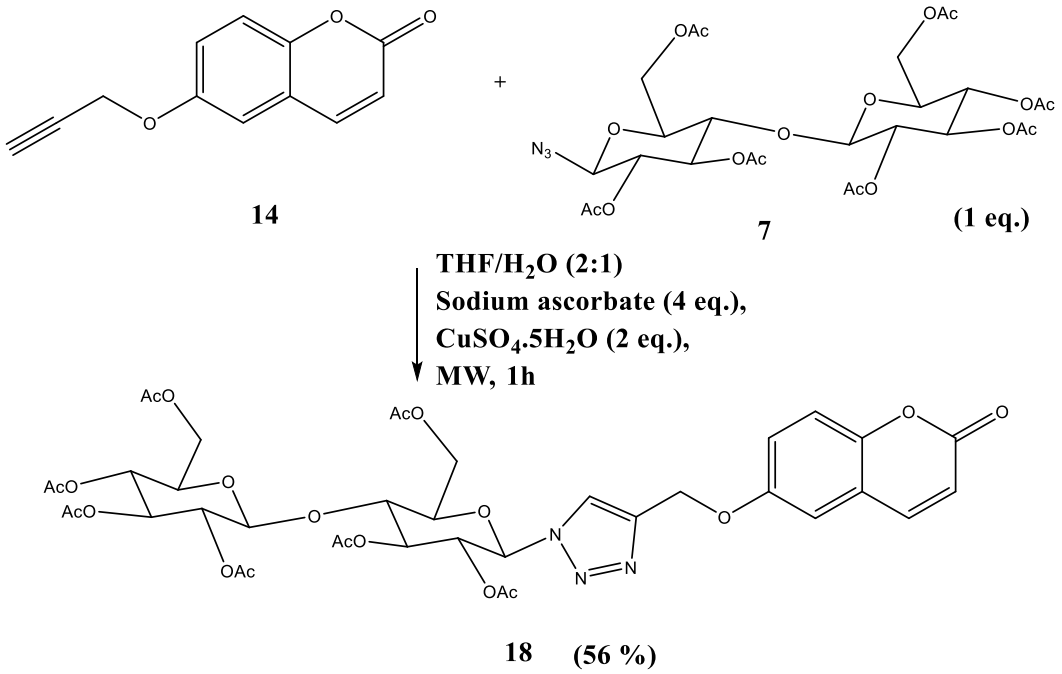


Figure 3.1. General equation for a click reaction

The catalytic system of CuAAC generally consists of copper(II) salts with a reducing agent (such as sodium ascorbate or hydrazine hydrate).[4] Sodium ascorbate is able to

reduce copper(II) to copper(I) and replaces the oxygen-free conditions. The main disadvantage is that the reducing agent can reduce Cu (II) directly to Cu (0).[5]

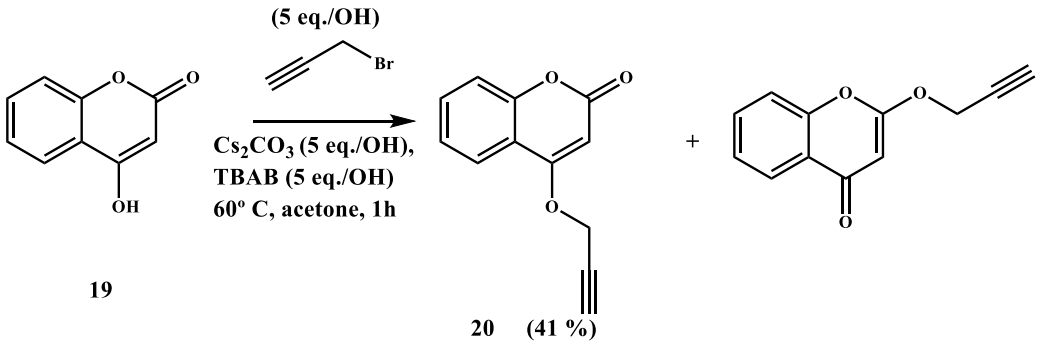
Therefore, for the first time, the 6-(1-(1(-*β*-D-heptacetocellobiosyl)-1*H*-1,2,3-triazole-6-yl)methoxy)-2*H*-chromene-2-one (**18**) (**Scheme 3.1**) was obtained by Cu (I)-catalyzed click chemistry of 6-(prop-2-yn-yloxy)coumarin (**14**) and a commercial *β*-D-heptacetocellobiosyl azide (**7**) (**Scheme 3.1**) carried out in the presence of copper (II) sulfate penta-hydrate and sodium ascorbate in a mixture of THF/water (2:1) for 1 hour at 70 °C under microwave irradiation at 300 W (η= 56 %).



Scheme 3.1. Synthesis of 6-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**18**).

3.1.1.2. Synthesis of 4-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**)

For the first time, the 4-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**) (**Scheme 3.2**) was obtained by reaction of 4-hydroxycoumarin (**19**) with propargyl bromide in acetone (η= 41 %).

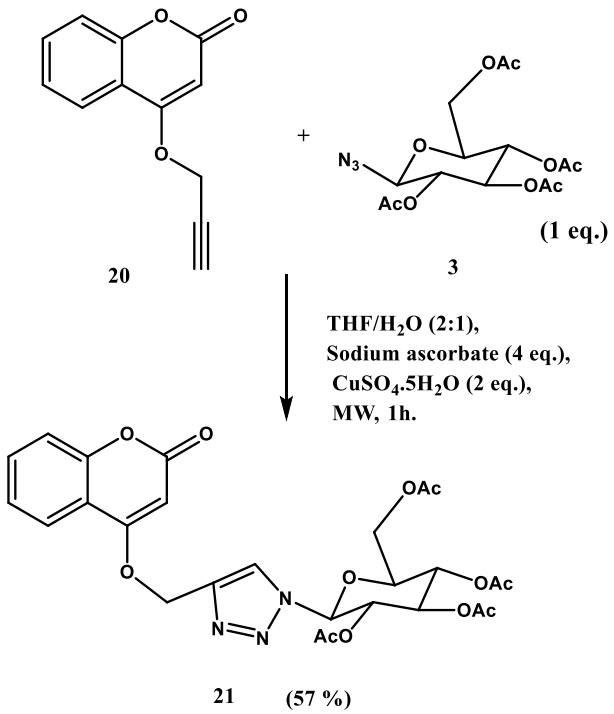


Scheme 3.2. Synthesis of 4-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**).

The bimolecular nucleophilic substitution allows an exchange of substituent groups, involving an attack of a Lewis base or nucleophile to a Lewis acid or electrophile. In basic conditions, the hydroxyl groups of 4-hydroxycoumarin (**19**) are deprotonated and act as nucleophiles. Alkyl halides such as propargyl bromide acts as electrophiles since they good leaving groups and are attacked by nucleophiles such as **19**, suffering a halogen substitution.

3.1.1.3. Synthesis of 4-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**21**)

For the first time, the 4-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-*D*-glucopyranosyl)1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**21**) (**Scheme 3.3**) was obtained by reaction Cu (I)-catalyzed click chemistry of 4-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**) and *β*-glucopyranosyl azide tetraacetate **3** carried out in the presence of copper (II) sulfate pentahydrate and sodium ascorbate in a non-homogeneous mixture of THF/water (2:1) for 1 hour at 70 °C under MW at 300 W (η= 57 %).



Scheme 3.3. Synthesis of 4-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**21**).

3.1.2. Structure elucidation

3.1.2.1. 6-(1-(1-(2,3,6,2',3',4',6'-Hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**18**)

The structure of 6-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**18**) (**Figure 3.2**) was established by IR (**Figure 3.3**), ^{1}H NMR (**Figure 3.4**), ^{13}C NMR (**Figure 3.5**), and HSQC/HMBC techniques (**Figure 3.6**).

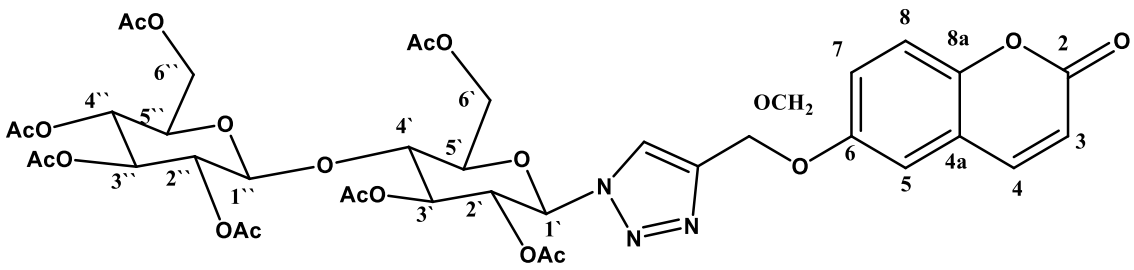


Figure 3.2. Structure of 6-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**18**).

The IR spectra of compound **18** is presented in figure and shows a band at 1750 cm^{-1} associated with C=O ester bond (**Figure 3.3**).

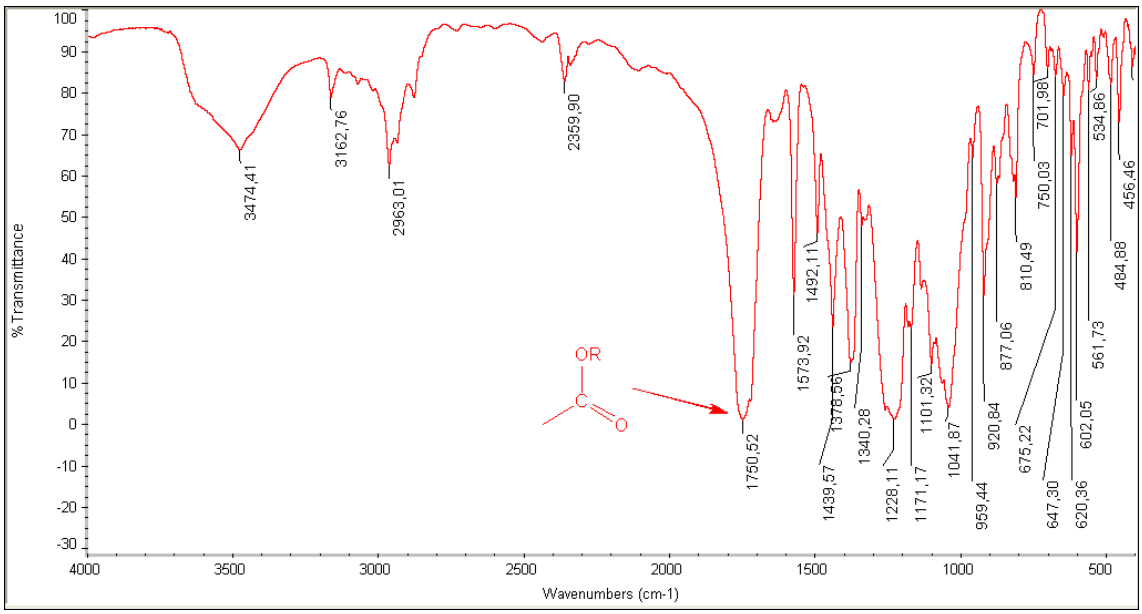


Figure 3.3. IR spectra (KBr) of the 6-cellobiosyl triazole coumarin **18**

The ^{1}H and ^{13}C NMR data of compound **4** are presented in **Table 3.1**.

Table 3.1. ^{1}H and ^{13}C NMR data (DMSO-d_6) of the 6-cellobiosyl triazole coumarin **18**.

^{1}H	δ (ppm)	^{13}C	δ (ppm)
	4		4
H-triazole	9.48 (1H, s)	***C*OOCH$_3$**	170.3
H-4	7.99 (1H, d, *J*=9.5 Hz)	***C*OOCH$_3$**	170.1
H-5	7.39 (1H, d, J=9.5 Hz)	***C*OOCH$_3$**	169.6
H-7	7.36 (1H, d, J=9.0 Hz)	***C*OOCH$_3$**	169.4
H-8	7.27 (1H, dd, J=9.6 and 2.9 Hz)	***C*OOCH$_3$**	169.2
H-3	6.50 (1H, d, J=9.5 Hz)	***C*OOCH$_3$**	169.0
H-1`	6.28 (1H, d, J= 9.2 Hz)	***C*OOCH$_3$**	168.5
H-cellobiose	5.53 (1H, J= 9.4 Hz)	**C-2**	160.1
H-cellobiose	5.45 (1H, t, J= 9.5 Hz)	**C-6**	154.2
H-cellobiose	5.30 (1H, t, J= 9.6 Hz)	**C-8a**	148.1
OC*H$_2$*	5.20 (2H, s)	**C-4**	144.1

H-cellobiose	4.91 (2H, t, J= 9.3 Hz)	***C*=CH-triazole**	143.1
H-cellobiose	4.68 (1H, dd, J= 9.6 and 8.1 Hz)	***C*H=C-triazole**	123.8
H-1``	4.40 (1H, d, J= 11 Hz)	**C-4a**	120.1
H-cellobiose	4.28 - 4.22 (2H, m)	**C-8**	119.2
H-Cellobiose	4.09 3.93 (5H, m)	**C-7**	117.5
COOC*H*$_3$	2.04 - 1.90 (21H, m)	**C-1`**	116.7
		C-5	112.2
		C-3	99.6
		C-1``	83.6
		C-cellobiose	76.0
		C-cellobiose	74.4
		C-cellobiose	72.2
		C-cellobiose	72.0
		C-cellobiose	71.8
		C-cellobiose	70.4
		C-cellobiose	70.2
		C-cellobiose	67.6
		C-cellobiose	62.2
		C-cellobiose	61.5
		O-*C*H$_2$	61.4
		COO*C*H$_3$	20.6
		COO*C*H$_3$	20.4
		COO*C*H$_3$	20.3
		COO*C*H$_3$	20.2
		COO*C*H$_3$	20.1
		COO*C*H$_3$	19.9

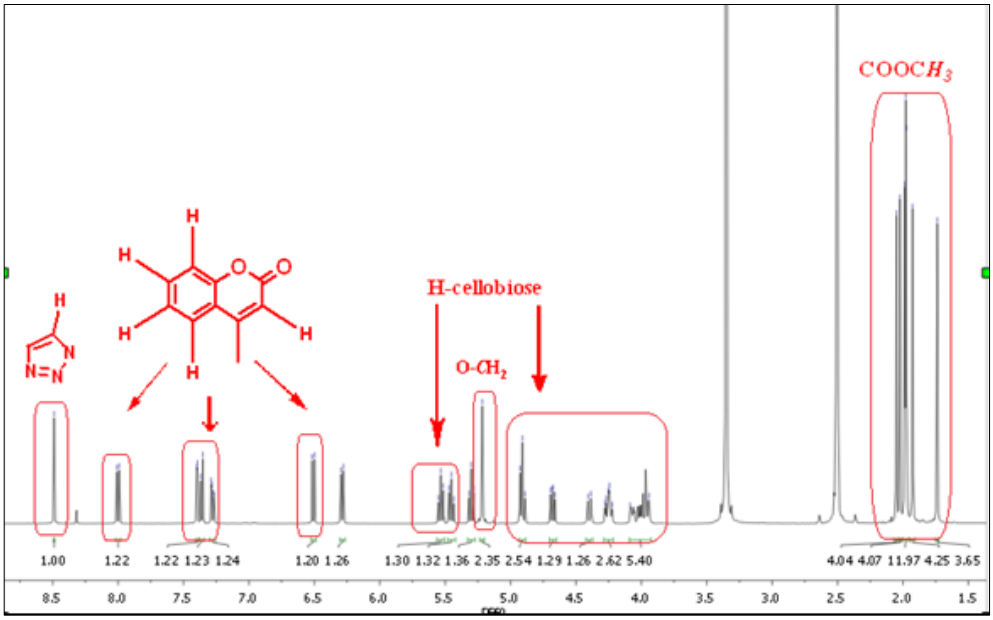


Figure 3.4. ^{1}H NMR spectra (DMSO-d_6) of the 6-cellobiosyl triazole coumarin **18**.

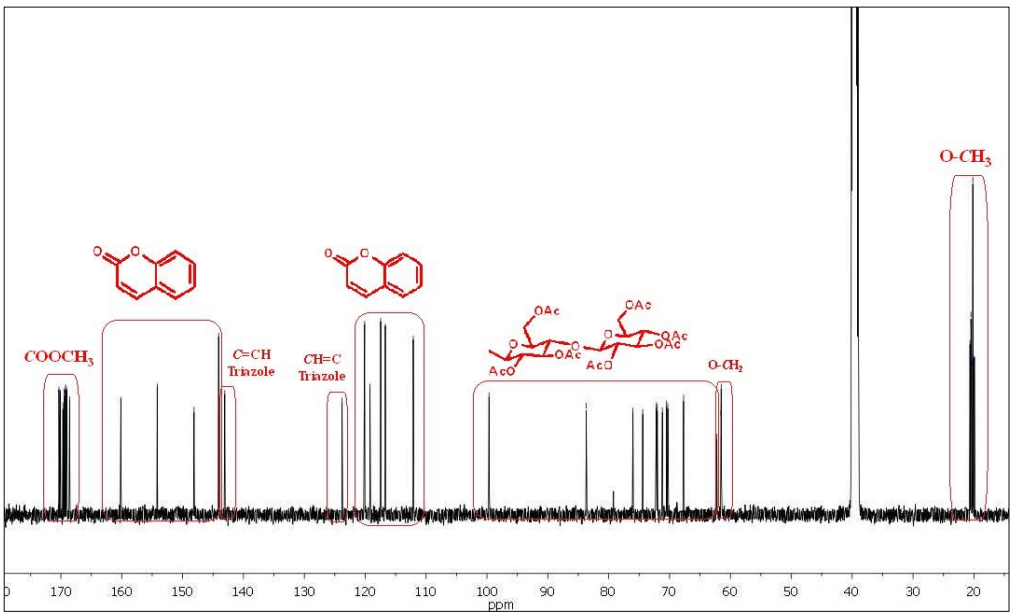


Figure 3.5. ^{13}C NMR spectra (DMSO-d_6) of the 6-cellobiosyl triazole coumarin **18**.

In the ^{1}H of compound **18**, a chemical shift δ_H 8.48 ppm (1H, s, H-triazole) and other δ_H 5.24 ppm (2H, s, OC*H*$_2$) are observed, which confirms the presence of a triazole ring (**Figure 3.4**). The ^{13}C NMR showed also the chemical shifts δ_C 143.1 ppm (*C*=CH-triazole), δ_C 123.8 (*C*H=C-triazole), and δ_C 61.4 ppm (O*C*H$_2$) corresponding to the carbons of the triazole ring (**Figure 3.5**). With HMBC studies it was possible to confirm the attachment of the ether bound to the coumarin (**Figure 3.6**). This coumarin kept the saccharidic moiety in the *β* configuration (*J*=9.2 Hz, H-1`).[6]

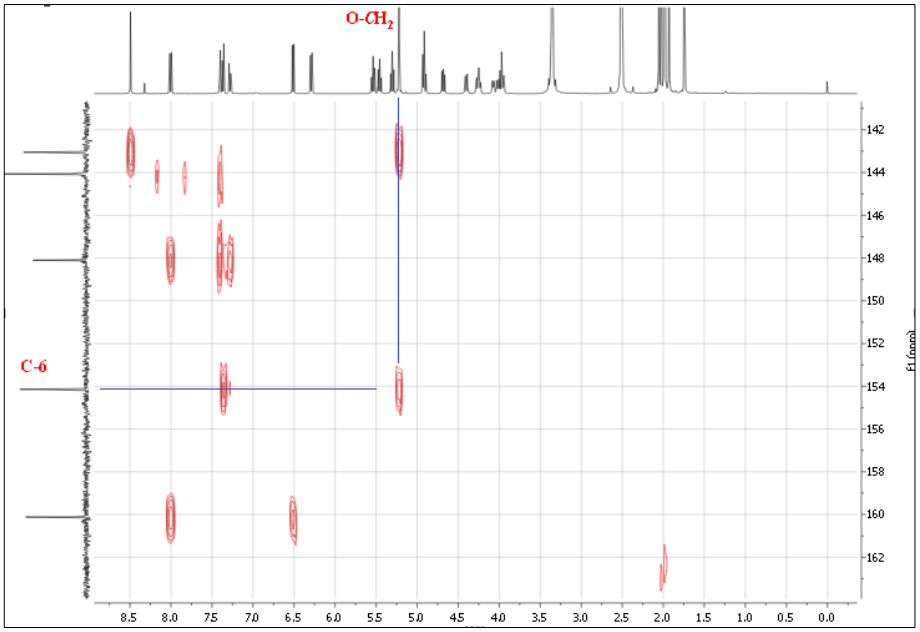


Figure 3.6. Heteronuclear multiple-bond correlation (DMSO-d_6) of the 6-cellobiosyl triazole coumarin **18**.

3.1.2.2. 4-(Prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**)

The structure of 4-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**) (**Figure 3.7**) was established by IR (**Figure 3.8**), ^{1}H NMR (**Figure 3.9**), and ^{13}C NMR (**Figure 3.10**).

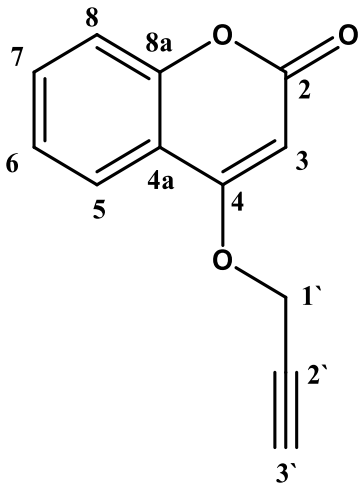


Figure 3.7. Structure of 4-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**).

The IR spectra of compound **20** shows a band at 3280 cm^{-1} confirming the presence of a triple bond (**Figure 3.8**).

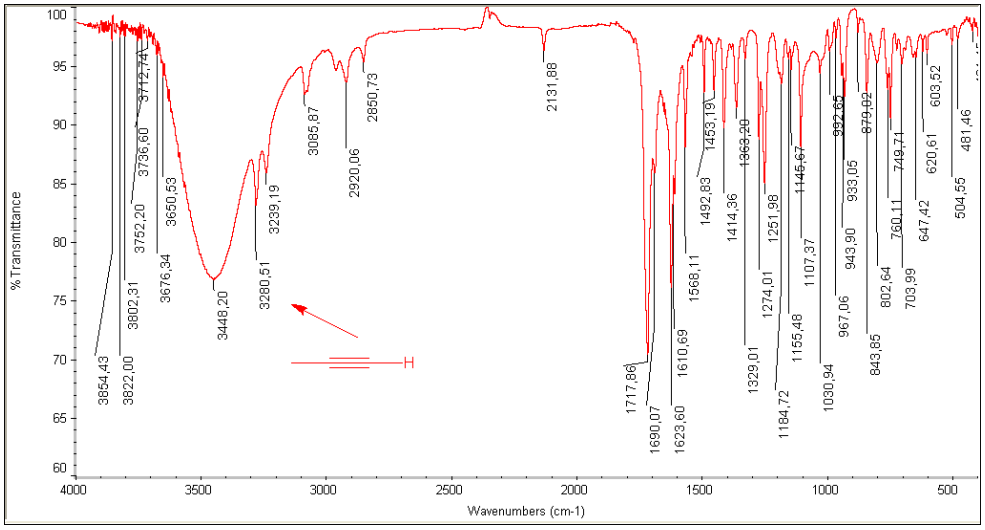


Figure 3.8. IR spectra (KBr) of the 4-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**).

The ^{1}H and ^{13}C NMR data (DMSO-d_6) of coumarins **19** and **20** are presented in **Table 3.2.**

Table 3.2. ^{1}H and ^{13}C NMR data (DMSO-d_6) of the 4-hydroxy-2*H*-chromene-2-one (**19**) and 4-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**).

^{1}H	δ (ppm)		^{13}C	δ (ppm)	
	19	**20**		**19**	**20**
5	7.82 (d, J=1.5 Hz)	7.79 (dd, J=9.6 and 7.9 Hz)	**4**	165.5	163.7
7	7.64 (t, J=1.4 Hz)	7.70 - 7.65 (m)	**2**	161.8	161.3
8	7.39 (d, J=2.9 Hz)	7.42 - 7.33 (m)	**8a**	153.5	152.8
6	7.35 (t, 1.7 Hz)		**5**	132.9	123.0
3	5.60 (s)	5.96 (s)	**7**	132.6	133.0
1`		4.86 (J=2.3 Hz)	**6**	123.1	124.4
3`		5.11 (s)	**4a**	116.7	115.0
			8	116.3	117.0
			3	90.9	96.1
			2`		80.2
			3`		77.2
			1`		57.4

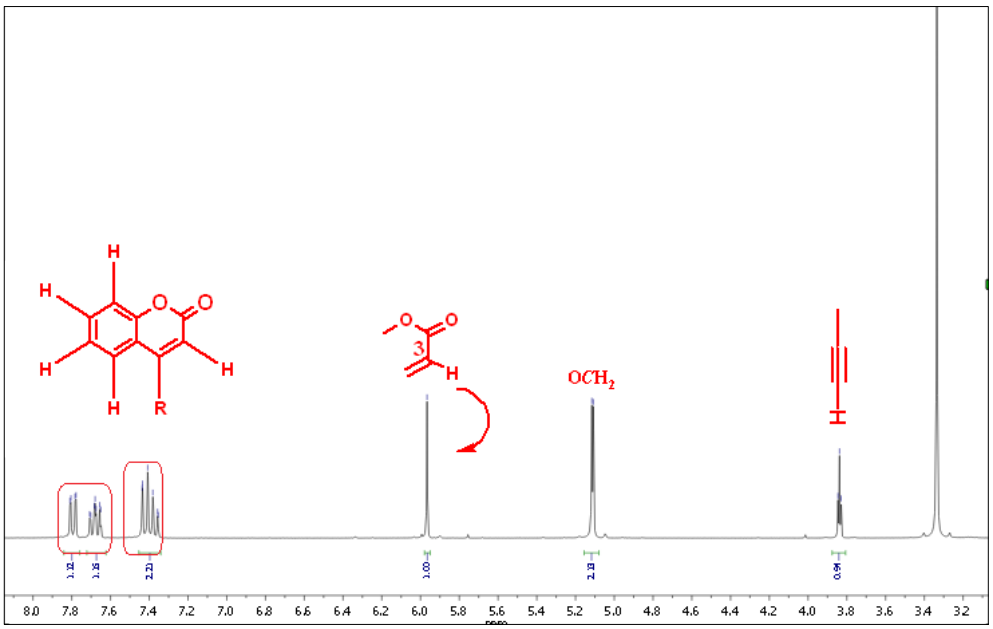


Figure 3.9. ^{1}H NMR spectra (DMSO-d_6) of the 4-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**).

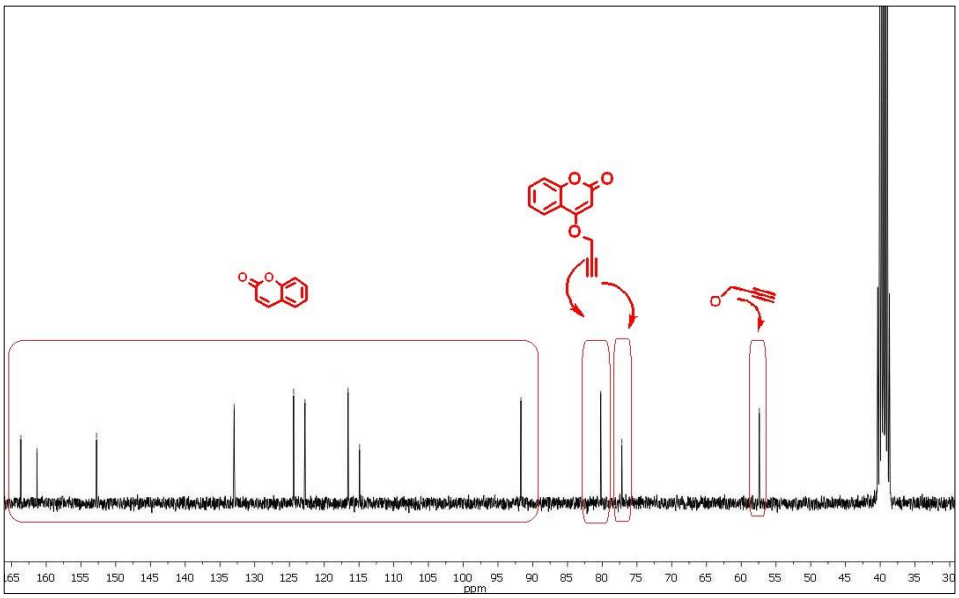


Figure 3.10. ^{13}C NMR spectra (DMSO-d_6) of the 4-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**).

Two additional signals appear in the proton spectra as well as three new signals in the carbon spectrum corresponding to the propargyl moiety (**Figure 3.9** and **3.10**). The proton of the 4-hydroxy-2*H*-chromene-2-one phenol is not visible due to hydrogen bonds.[7]

3.1.2.3. 4-(1-(1-(2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**21**)

The structure of 4-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**21**) (**Figure 3.11**) was established by IR (**Figure 3.12**), ^{1}H (**Figure 3.13**), ^{13}C NMR (**Figure 3.14**) and HSQC/HMBC techniques (**Figure 3.15**).

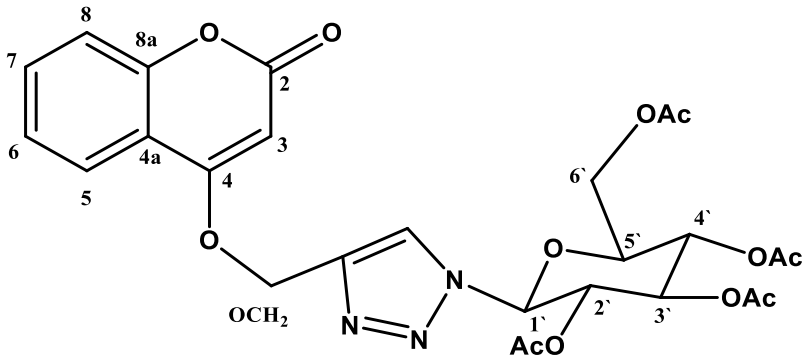


Figure 3.11. Structure of 4-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**21**)

The IR spectra of compound **21** is presented in figure and shows a band at 1751 cm^{-1} associated with C=O ester bond (**Figure 3.12**).

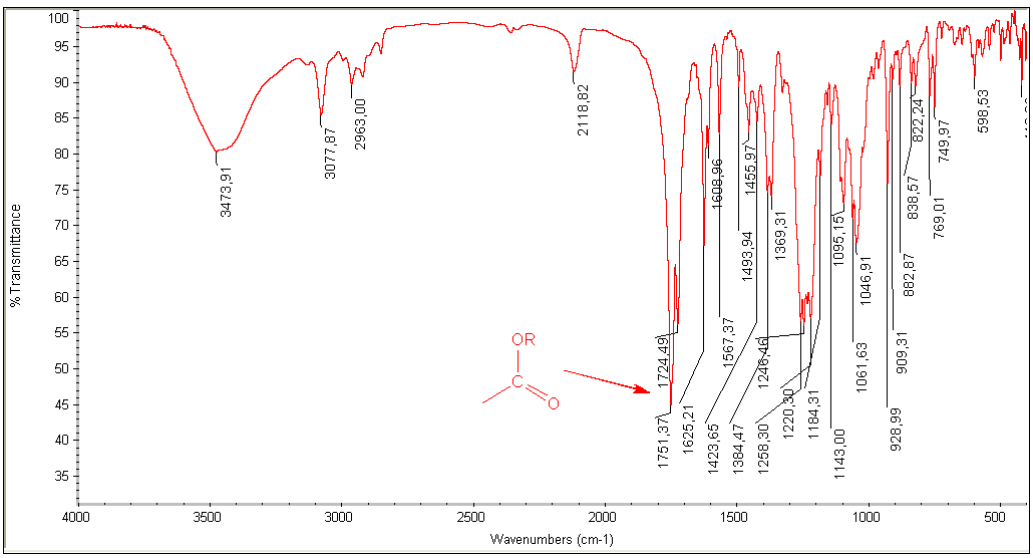


Figure 3.12. IR spectra (KBr) of the 4-glycosyl triazole coumarin **21**.

The ^{1}H and ^{13}C NMR data of compound **21** are presented in **Table 3.3.**

Table 3.3. ^{1}H and ^{13}C NMR data (DMSO-d_6) of the 4-glycosyl triazole coumarin **21**.

^{1}H	δ (ppm)	^{13}C	δ (ppm)
	21		**21**
H-triazole	8.71 (1H, s)	**COOCH$_3$**	170.3
H-5	7.78 (1H, dd, J=7.9 and 1.5 Hz)	**COOCH$_3$**	170.1
H-7	7.70 – 7.64 (1H, m)	**COOCH$_3$**	169.6
H-6 and H-8	7.44 – 7.32 (2H, m)	**COOCH$_3$**	169.4
H-1`	6.42 (1H, d, J= 9.1 Hz)	**C-4**	164.2
H-3	6.12 (1H, s)	**C-2**	161.5
H-glucose	5.60 (1H, d, J= 9.4 Hz)	**C-8a**	152.8
OCH$_2$	5.47 (2H, s)	**C=CH-triazole**	142.0
H-glucose	5.21 (1H, t, J= 9.7 Hz)	**C-7**	132.8
H-glucose	4.13 (2H, t, J= 5.1 Hz)	**C-6**	124.2
OCH$_2$	2.07 – 1.95 (7H, m)	**C-5**	124.1
H-glucose	1.78 (3H, s)	**CH=C-triazole**	122.8
		C-8	116.5
		C-4a	115.0
		C-1`	91.5
		C-3	86.2
		C-glucose	83.9
		C-glucose	73.3
		C-glucose	72.0
		C-glucose	67.5
		C-glucose	62.7
		OCH$_2$	61.7
		COOCH$_3$	20.5
		COOCH$_3$	20.4
		COOCH$_3$	20.2
		COOCH$_3$	19.8

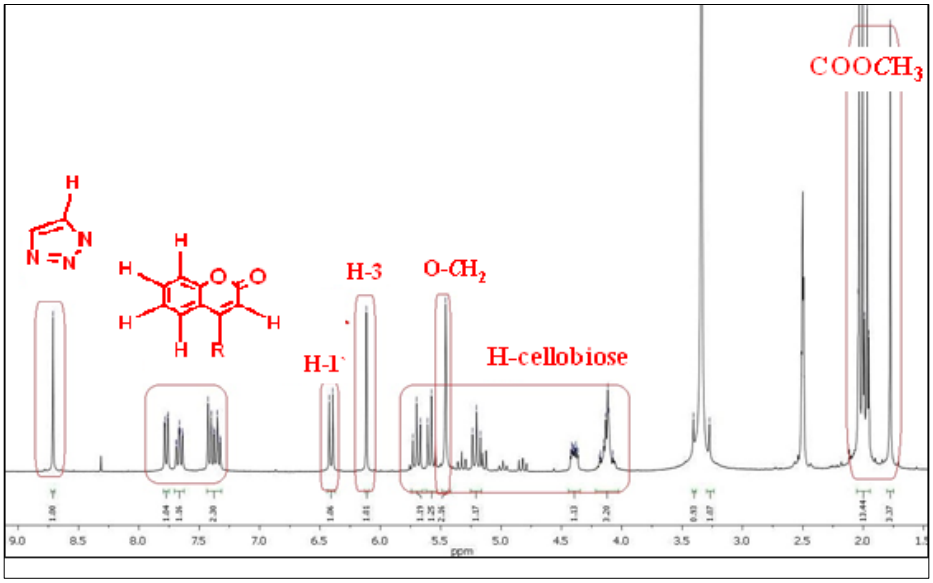


Figure 3.13. ^{1}H NMR spectra (DMSO-d$_6$) of the 4-glycosyl triazole coumarin **21**.

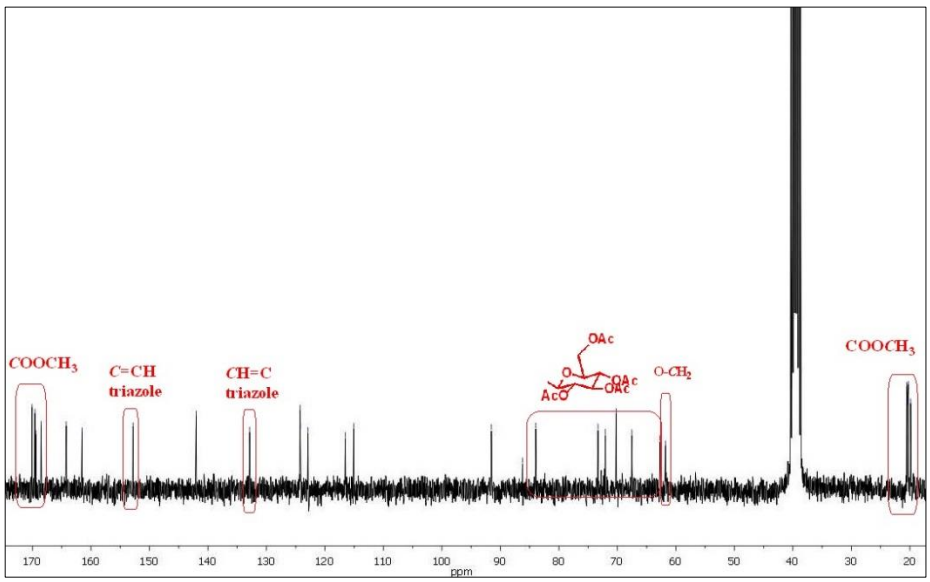


Figure 3.14. ^{13}C NMR spectra (DMSO-d$_6$) of the 4-glycosyl triazole coumarin **21**.

In the ^{1}H NMR spectra of compound **21**, a signal at δ_H 8.71 ppm (^{1}H, s, *H*-triazole) and other at 5.40 ppm (2H, s, OC*H*$_2$) are observed, which confirms the presence of a triazole ring (**Figure 3.13**). The ^{13}C NMR showed also δ_C 143.1 ppm (*C*=CH-triazole), δ_C 123.8 (*C*H=C-triazole), and δ_C 61.4 ppm (O*C*H$_2$) corresponding to the carbons of the triazole ring (**figure 3.14**). In this glycosyl triazole coumarin remains the *β* conformational according to the constant coupling (*J*=9.1 Hz, H-1`).

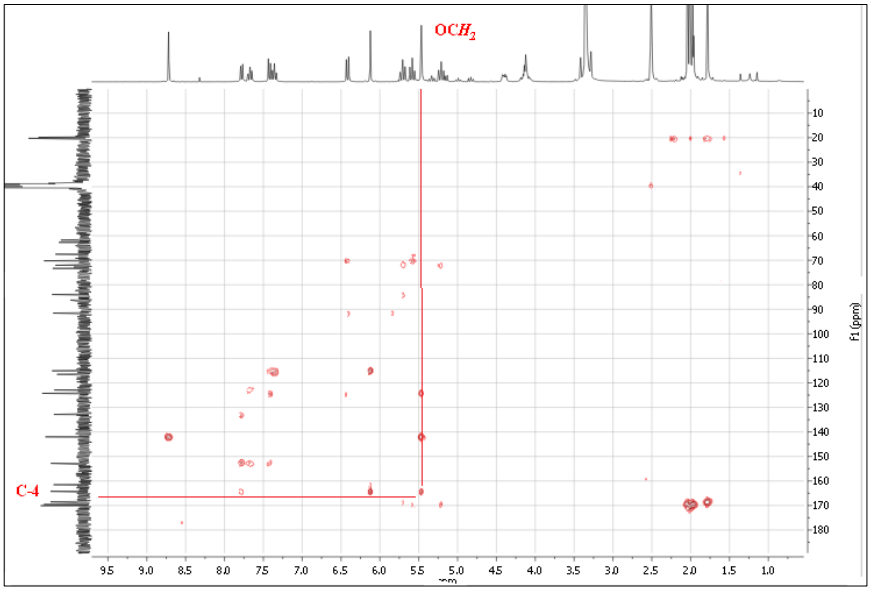


Figure 3.15. Heteronuclear multiple bond correlation of the 4-glycosyl triazole coumarin **21**.

3.2. Experimental section

3.2.1. Materials and methods

This content was previously reported on chapter 2, section 2.3.1.

3.2.2. Synthesis

6-(1-(1-(2,3,6,2',3',4',6'-Hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)-2*H*-chromene-2-one (**18**)

6-(1-(1-(2,3,6,2',3',4',6'-Hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy) -2*H*-chromene-2-one (**18**) was prepared from 6-(prop-2-yn-yloxy)-2*H*-chromene-2-one (**14**) (200 mg, 1.00 mmol) in similar conditions than 4-methyl-(1-(1-(2,3,6,2',3',4',6'-hepta-*O*-acetyl-*β*-D-cellobiosyl)-1*H*-1,2,3-triazole-4-yl)methoxy) umbelliferone (**8**) to give a desired product as a yellow solid (479 mg, 56 %). The chromatographic control was performed by TLC (silica gel, chloroform/acetone, 90:10).

M.p.: 244 – 246 °C. IR umax (cm^{-1}) (KBr): 3480; 3160; 2970; 1750; 1573. ^{1}H-NMR (DMSO-d_6,500.16 MHz) δ (ppm): 8.48 (1H, s, H-triazole), 7.99 (1H, d, *J*=9.5, H-4), 7.39 (1H, d, *J*=2.9 Hz, H-5), 7.36 (1H, d, *J*=9.0 Hz, H-7), 7.27 (1H, dd, *J*=9.6 and 2.95 Hz, H-8), 6.50 (1H, d, *J*=9.5 Hz, H-1`), 6.28 (1H, d, *J*=9.2 Hz, H-3), 5.53 (1H, t, *J*=9.4 Hz, H-cellobiose), 5.45 (1H, t, *J*=9.5 Hz, H-cellobiose), 5.30 (1H, t, *J*=9.6 Hz, H-cellobiose), 5.20 (2H, s, OCH_2), 4.91 (2H, t, *J*=9.3 Hz, H-cellobiose), 4.69 - 4.65 (1H, m, H-cellobiose) 4.39 (1H, d, *J*=11.1

Hz, H-cellobiose), 4.27 – 4.22 (1H, m, H-cellobiose), 2.04 – 1.90 (18H, m, COOC*H*$_3$), 1.78 (3H, s, COOC*H*$_3$). ^{13}C-NMR (DMSO-d$_6$, 125.77 MHz) δ (ppm): 170.3 (*C*OOCH$_3$), 170.1 (*C*OOCH$_3$), 169.6 (*C*OOCH$_3$), 169.4 (*C*OOCH$_3$), 169.2 (*C*OOCH$_3$), 169.0 (*C*OOCH$_3$), 168.5 (*C*OOCH$_3$), 160.1 (C-2), 154.2 (C-6), 148.1 (8a), 144.1 (C-4), 143.1 (*C*=CH triazole), 123.8 (*C*H=C triazole), 120.1 (C-4a), 119.2 (C-8), 117.5 (C-7), 116.7 (C-3), 112.2 (C-5), 99.6 (C-1``), 83.6 (C-1`), 76.0 (C-cellobiose), 74.4 (C-cellobiose), 72.2 (C-cellobiose), 72.0 (C-cellobiose), 71.8 (C-cellobiose), 70.4 (C-cellobiose), 70.2 (C-cellobiose), 67.6 (C-cellobiose), 62.2 (C-cellobiose), 61.5 (C-cellobiose), 61.4 (O*C*H$_2$), 20.6 (COO*C*H$_3$), 20.4 (COO*C*H$_3$), 20.3 (COO*C*H$_3$), 20.2 (COO*C*H$_3$), 20.1 (COO*C*H$_3$), 19.9 (COO*C*H$_3$).

4-(Prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**)

4-(Prop-2-yn-yloxy)-2*H*-chromene-2-one (**20**) was prepared from 4-hydroxy-2*H*-chromene-2-one (**19**) (1000 mg, 6.17 mmol) in similar conditions than 6-(prop-2-yn-yloxy)coumarin (**14**) to obtain the desired product as a white solid (226 mg, 41 %). The chromatographic control was performed by TLC (silica gel, n-hexane/ethyl acetate, 70:30).

M.p.: 161 – 163 °C. IR υmax (cm^{-1}) (KBr): 3281, 3239, 1718. ^{1}H-NMR (DMSO-d$_6$,300.13 MHz) δ (ppm): 7.79 (1H, dd, *J*=1.5 and 7.9 Hz, H-5), 7.70 – 7.65 (1H, m, H-7), 7.42 – 7.33 (2H, m, H-8, H-6), 5.96 (1H,s, H-3), 5.11 (2H, *J*=2.4 Hz, OC*H*$_2$), 3.83 (1H, t, *J*=2.4 Hz, H-3`). ^{13}C-NMR (DMSO-d$_6$, 75.47 MHz) δ (ppm): 163.7 (C-4), 161.3 (C-2), 152.8 (C-8a), 133.0 (C-7), 124.4 (C-6), 123.0 (C-5), 117.0 (C-8), 115.0 (C-4a), 91.6 (C-3), 80.2 (C-alkyne), 77.2 (*C*H-alkyne), 57.4 (O*C*H$_2$).

4-(1-(1-Tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy) -2*H*-chromene-2-one (**21**)

4-(1-(1-(2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl) methoxy) -2*H*-chromene-2-one (**21**) was prepared from of 4-(prop-2yn-yloxy)coumarin (**20**) (200 mg, 1.00 mmol) in similar conditions than 4-methyl-(1-(1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)umbelliferone (**4**) to give a desired product as a white solid (329 mg, 57 %). The chromatographic control was performed by TLC (silica gel, chloroform/acetone, 90:10)

M.p.: 246 - 247 °C. IR υmax (cm^{-1}) (KBr): 3474, 2119, 1751. ^{1}H-NMR (DMSO-d$_6$,300.13 MHz) δ (ppm): 8.71 (1H, s, H-triazole), 7.77 (1H, dd, *J*=1.4 and *J*=1.4 Hz, H-5) 7.69 – 7.63 (1H, m, H-7) 7.42 – 7.32 (2H, m, H-8, H-6), 6.41 (1H, d, *J*=9.1 Hz, H-1`), 6.11

(1H, s, H-3), 5.70 (1H, t, *J*=9.3 Hz, H-4`), 5.57 (1H, t, *J*=9.5 Hz, H-3`), 5.45 (1H, s, OCH_2), 5.23 – 5.15 (1H, m, H-2`), 4.42 – 4.36 (1H, m, H-5`), 4.18 – 4.07 (3H, m, H-6`), 2.04 – 1.95 (6H, m, COOCH_3), 1.77 (3H, s, COOCH_3). ^{13}C-NMR (DMSO-d_6, 75.47 MHz) δ (ppm): 170.1 (1 *C*OOCH$_3$), 169.8 (1 *C*OOCH$_3$), 169.4 (1 *C*OOCH$_3$), 168.5 (1 *C*OOCH$_3$), 164.2 (C-4), 161.5 (C-2), 152.8 (C-8a), 142.0 (*C*=CH triazole), 132.9 (C-7), 124.3 (*C*H=C triazole), 124.2 (C-6), 122.8 (C-5), 116.5 (C-8), 115.1 (C-4a), 91.5 (C-3), 83.9 (C-1`), 73.3 (C-5`), 72.0 (C-3`), 70.0 (C-4`), 67.5 (C-2`), 62.7 (C-6`), 61.8 (O*C*H$_2$), 20.5 (COO*C*H$_3$), 20.4 (COO*C*H$_3$), 20.2 (COO*C*H$_3$), 19.9 (COO*C*H$_3$).

3.3. References

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Chapter 4 - Anticoagulant activity evaluation of another sulfated compound

4. Anticoagulant activity evaluation of another sulfated compound

The anticoagulant activity of *in house* synthesized glycosylated ascorbic acid persulfate **22** (**Figure 4.1**) was also tested in classical coagulation assays along with the derivatives investigated in chapter 2. This study is related to another ongoing project at LQOF.

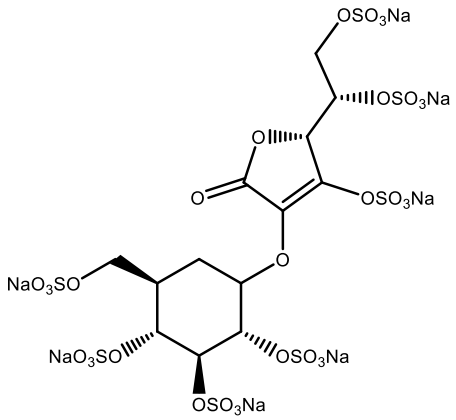

Figure 4.1. Structure of persulfated glycosyl ascorbic acid **22**.

4.1. Clotting times

The anticoagulant activity of sulfated compound **22** was measured in human plasma by the classical clotting times - activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) – in five different concentrations (**Figure 4.2**). The concentrations needed to double each clotting time are summarized in **Table 4.1.**

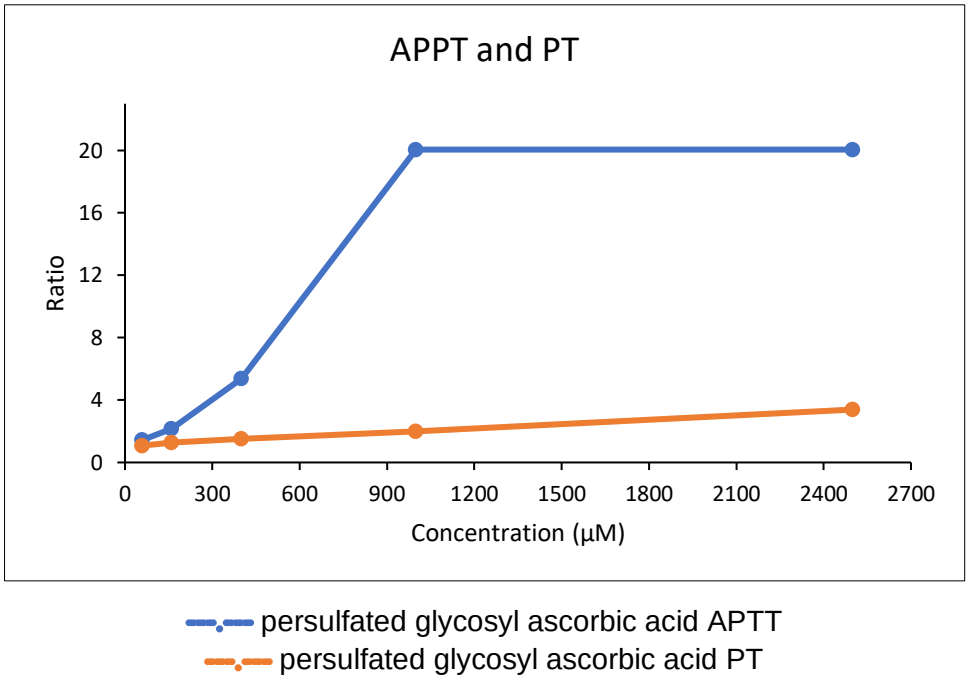

Figure 4.2. Dose-dependent effects of compound **22** on APTT and PT clotting assays using human pooled plasma, expressed as ratio of clotting time.

Table 4.1. Concentrations of persulfated compound **22** required to double clotting assays.

	$APTT_2$	PT_2	TT_2
22	**137 ± 6**	**1023 ± 107**	**N.A.**

N.A. not active at 2.5×10^{-3} M

The tested sulfated compound **22** showed anticoagulant properties in a dose dependent manner. The APTT was the most sensitive test to the presence of this sulfated compound which were able completely block this clot formation pathway at the highest concentration (2500 µM). Heparins prologue the APTT assays so possibly this compound forms a complex with AT III, this complex inhibits the formation of thrombin and accelerates its destruction. In addition, may also inactivate other proteases from the coagulation cascade, especially FXa.

4.2. Biological activity

This content was previously reported on chapter 2, section 2.3.3.

Chapter 5 – Conclusions

5. Conclusions

Over the last 15 years, according to the World Health Organization the world's leading causes of death are ischemic heart disease and stroke, which constitute the number one and two of the top 10 global causes of death and accounted for a combined 15.2 million deaths in 2016. For many years heparins and vitamin K antagonists ruled over anticoagulant therapy however, with a number of drawbacks associated. The new oral anticoagulants have solved some previously issues, but some concerns still prevail due to some side effects.

In this work, seven compounds coumarin derivatives were synthesized **2**, **8**, **9**, and news **10**, **18**, **20** and **21**. The anticoagulant activity of the sulfated coumarin and other sulfated compound were evaluated in human plasma.

Compounds **6** and **17** (6- and 7- glycosyl coumarins persulfates both with triazole) only affected the APTT assay ($APTT_2$ = ± 600 μM). These glycosyl coumarins showed similar double concentrations, allowing to hypothesize that the position of the saccharidic moiety had poor influence on activity. When comparing 7-glycosyl coumarin **12** and 7-glycosyl triazole coumarin **10** ($APTT_2$ = ± 200 μM) it can be concluded that the presence of triazole has no influence on coagulation. Nevertheless, the triazole can enhance the stability between glycosidic moiety and coumarin as well as improve oral bioavailability. Comparing 7-cellobiosyl triazole coumarin **10** ($APTT_2$ = ± 200 μM) and 7-glycosyl triazole coumarin **6** ($APTT_2$ = ± 600 μM) it can be concluded that saccharide cellobiose has a great influence on anticoagulant activity.

By adding sulfated saccharides to the coumarin scaffold it was possible to obtain coumarins with an anticoagulant profile similar to the anticoagulant heparin (in general, the APTT was more affected by these compounds than PT), however with higher potential to be oral bioavailable. Recently, attention has been drawn to the nonanticoagulant activities of heparin. On the molecular level, heparin inhibits the function, expression and/or synthesis of adhesion molecules, cytokines, angiogenic factors which confers a polypharmacological mode of action.

Further ahead *in vivo* studies will be conducted to prove the efficacy of these compounds. We hope that these hybrids might lead to new antithrombotic agents mimetic of heparin but with oral availability.