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Potential Orally-Active Heparin-Like Compounds: Synthesis and Anticoagulant Activity

Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Química Farmacêutica

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* Presenting author
If you want to go fast go alone,
If you want to go far go together.

African Proverb
INDEX

FIGURES INDEX ........................................................................................................ XIII
TABLE INDEX ........................................................................................................ XV
SCHEME INDEX ...................................................................................................... XVII
AGRADECIMENTOS ................................................................................................. XIX
ABSTRACT ................................................................................................................ XXI
RESUMO .................................................................................................................... XXIII
ABBREVIATIONS ..................................................................................................... XXV
OUTLINE OF THE DISSERTATION .......................................................................... XXVII

CHAPTER 1 - INTRODUCTION ................................................................................. 1

1.1. Oral bioavailability of drugs ............................................................................. 1
1.2. Strategies to obtain orally-active heparins ....................................................... 2
  1.2.1. Drug conjugates ............................................................................................ 4
    1.2.1.1. Heparin-deoxycholic acid conjugates .................................................... 4
    1.2.1.2. Heparin-fatty acids and heparin-cholesterol conjugates ....................... 11
  1.2.2. Formulations with penetration enhancers .................................................... 13
    1.2.2.1. Permeation enhancers .......................................................................... 14
    1.2.2.2. Absorption enhancers .......................................................................... 17
  1.2.3. Micro and nanotechnology ......................................................................... 19
1.3. Aims: development of polysulfated small-molecules towards new oral antithrombotic agents ........................................................................................................ 20

CHAPTER 2 - RESULTS AND DISCUSSION .......................................................... 23

2.1. SYNTHESIS ....................................................................................................... 25
  2.1.1. Conjugation of mangiferin with deoxycholic acid ....................................... 25
    2.1.1.1. Acetylation of mangiferin (5) .................................................................. 25
    2.1.1.2. Carbomethoxymethylation of mangiferin (5) ......................................... 27
  2.1.2. Conjugation of naringin with deoxycholic acid (I) ...................................... 28
  2.1.3. Conjugation of naringin with deoxycholic acid (II) ..................................... 30
    2.1.3.1. Carbomethoxymethylation of naringin (8) ............................................. 30
    2.1.3.2. Deacetylation of methyl 4’-naringin acetate (11) .................................... 30
2.1.3.3. Synthesis of succinimido deoxycholate (13) ........................................... 31
2.1.3.4. Synthesis of N-deoxycholyethylene diamine (14) ................................. 32
2.1.3.5. Synthesis of 4′-naringin (N-(2-deoxycholan-24-amidoethyl))-acetamide (15) 32
2.1.3.6. Sulfation of 4′-naringin (N-(2-deoxycholan-24-amidoethyl))-acetamide (15) 33
2.1.4. Glycosylation of 3,6-dihydroxy xanthone through a triazole .......................... 35
  2.1.4.1. Copper(I)-catalyzed alkyne-azide cycloaddition ...................................... 35
  2.1.4.2. Deacetylation of 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (19) ............ 35
  2.1.4.3. N-deacetylation of 3,6-bis(1-(2-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (20) ............................. 36
  2.1.4.4. Sulfation of 3,6-bis(1-(2-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (21) ......................... 37
2.1.5. Others ........................................................................................................ 38
  2.1.5.1. Acetylation of diosmin (23) ................................................................... 38
  2.1.5.2. Acetylation of rutin (25) ..................................................................... 39
  2.1.5.3. Sulfation of naringin (8) ..................................................................... 40
2.2. STRUCTURE ELUCIDATION ........................................................................... 41
  2.2.1. Mangiferin peracetate (6) ......................................................................... 41
  2.2.2. Mangiferin heptaacetate (7) .................................................................. 42
  2.2.3. Naringin-di-deoxycholate (10) ................................................................ 44
  2.2.4. Methyl 4′-naringin acetate (11) ............................................................... 45
  2.2.5. 4′-Naringin acetic acid (12) .................................................................. 48
  2.2.6. Succinimido deoxycholate (13) .............................................................. 49
  2.2.7. N-Deoxycholyethylene diamine (14) ...................................................... 50
  2.2.8. 4′-Naringin (N-(2-deoxycholan-24-amidoethyl))-acetamide (15) .............. 51
  2.2.9. 4′-Naringin (N-(2-deoxycholan-24-amidoethyl))-acetamide persulfate (16) . 52
  2.2.10. 3,6-Bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (19) ...................... 55
  2.2.11. 3,6-Bis(1-(2-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (20) .................................................. 56
2.2.12. 3,6-Bis(1-(2-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (21) ................................................................. 58

2.2.13. 3,6-Bis(1-(2-(2-amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (22) .................................................. 59

2.2.14. Diosmin peracetate (24) ........................................................................... 62

2.2.15. Rutin peracetate (26) ............................................................................... 63

2.2.16. Naringin persulfate (27) ........................................................................... 65

2.3. BIOLOGICAL ACTIVITIES ........................................................................... 68

2.3.1. Antitumor activity ..................................................................................... 68

2.3.2. Anticoagulant activity .............................................................................. 69

CHAPTER 3 - EXPERIMENTAL SECTION .............................................................. 76

3.1. GENERAL MATERIALS AND METHODS .................................................. 77

3.2. SYNTHESIS ................................................................................................. 78

3.2.1. Synthesis of mangiferin peracetate (6) ......................................................... 78

3.2.2. Synthesis of mangiferin heptaacetate (7) ..................................................... 78

3.2.3. Synthesis of naringin-di-deoxycholate (10) .............................................. 79

3.2.4. Synthesis of methyl 4'-naringin acetate (11) ............................................ 80

3.2.5. Synthesis of 4'-naringin acetic acid (12) ................................................... 80

3.2.6. Synthesis of succinimido deoxycholate (13) ............................................ 81

3.2.7. Synthesis of N-deoxycholylethylenediamine (14) ................................... 82

3.2.8. Synthesis of 4’-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15) 82

3.2.9. Synthesis of 4’-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide persulfate (16) ........................................................................................................ 83

3.2.10. Synthesis of 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (19) .................. 84

3.2.11. Synthesis of 3,6-bis(1-(2-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (20) ........................................... 85

3.2.12. Synthesis of 3,6-bis(1-(2-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (21) ......................... 85
3.2.13. Synthesis of 3,6-bis(1-(2-(2-amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (22) ........................................ 86
3.2.14. Synthesis of diosmin peracetate (24) ................................................................. 86
3.2.15. Synthesis of rutin peracetate (26) ................................................................. 87
3.2.16. Synthesis of naringin persulfate (27) ................................................................. 88

3.2. BIOLOGICAL ACTIVITY ......................................................................................... 89
  3.2.1. Anticoagulant activity ..................................................................................... 89
  3.2.2. Statistical analysis ....................................................................................... 91

CHAPTER 4 - CONCLUSIONS .................................................................................. 93

CHAPTER 5 - REFERENCES ....................................................................................... 97

CHAPTER 6 – APPENDICES .................................................................................... 107

APPENDIX I - ¹H and ¹³C NMR data for compounds 5 (DMSO-d₆), 6 (CDCl₃), and 7 (CDCl₃) ....................................................................................................................... 109
APPENDIX II - ¹H and ¹³C NMR data for compounds 8, 27, 11, and 12 (DMSO-d₆). . 111
APPENDIX III - ¹H and ¹³C NMR data for compounds 9 (DMSO-d₆), 13 (CDCl₃), and 14 (DMSO-d₆). .................................................................................................................... 113
APPENDIX IV - ¹H and ¹³C NMR data for compounds 10, 15, and 16 (DMSO-d₆). .... 115
APPENDIX V - ¹H and ¹³C NMR data for compounds 19, 20, 21, and 22 (DMSO-d₆). 117
APPENDIX VI - ¹H and ¹³C NMR data for compounds 23 (DMSO-d₆) and 24 (CDCl₃). ....................................................................................................................... 119
APPENDIX VII - ¹H and ¹³C NMR data for compounds 25 (DMSO-d₆) and 26 (CDCl₃). ....................................................................................................................... 121
FIGURES INDEX

Figure 1 – Unfractioned heparin (UFH) and low molecular weight heparins (LMWH)......... 3
Figure 2 - Advantages of heparins over other anticoagulant drugs. ........................................ 3
Figure 3 - Structural features of heparins that limit oral bioavailability. ................................. 4
Figure 4 – Deoxycholic acid (DOCA). ...................................................................................... 5
Figure 5 – Heparin-fatty acid conjugates. .................................................................................. 12
Figure 6 - Transcellular and paracellular absorption in the GI tract (adapted from 45) ....... 13
Figure 7 - Sodium caprate. ........................................................................................................ 14
Figure 8 - 18β-Glycyrrhetinic acid. ............................................................................................ 15
Figure 9 - Mono-N-carboxymethyl chitosan (MCC), a sulfonate derivative of N,O-carboxymethyl chitosan (SNOCC), and thiolated polycarbophil (PCP-Cys). ...................... 16
Figure 10 - Sodium N-(8-2-hydroxybenzoyl] amino) caprylate (SNAC) and sodium N-[10-(2-hydroxybenzoyl] amino] decanoate (SNAD). ................................................................. 16
Figure 11 - Polycationic lipophilic-core dendrons. ..................................................................... 17
Figure 12 – Examples of polysulfated glycosidic flavonoids/xanthonoids: rutin persulfate (1), 3,6-(O-β-glucopyranosyl) xanthon persulfate (2), mangiferin heptasulfate (3) diosmin persulfate (4). .................................................................................. 20
Figure 13 - Mangiferin (5). .......................................................................................................... 25
Figure 14 – Naringin (8). ............................................................................................................ 28
Figure 15 – Mangiferin peracetate (6). ....................................................................................... 41
Figure 16 – Mangiferin heptacacetate (7). .................................................................................. 42
Figure 17 – Naringin-di-deoxycholate (10). ................................................................................. 44
Figure 18 – Main connectivities found in HMBC for compound 10. ......................................... 45
Figure 19 – Methyl 4’-naringin acetate (11). ............................................................................. 45
Figure 20 – Main connectivities found in HMBC for compound 11. ......................................... 47
Figure 21 – 4’-Naringin acetic acid (12). ..................................................................................... 48
Figure 22 – Succinimido deoxycholate (DOCA-NHS, 13). ......................................................... 49
Figure 23 – N-Deoxycholylethylenediamine (DOCA-NH₂, 14). ................................................ 50
Figure 24 – 4’-Naringin (N-(2-deoxycholan-24-amidoethyl)acetamide (15). .......................... 51
Figure 25 – 4’-Naringin (N-(2-deoxycholan-24-amidoethyl)acetamide persulfate (16).... 52
Figure 26 - Main connectivities found in HMBC for compound 16. ........................................... 54
Figure 27 – 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthon (19). ......................................................... 55
Figure 28 – Main connectivities found in HMBC for compound 19. .......................................... 56
Figure 29 – 3,6-Bis(1-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (20). .......................................................... 57
Figure 30 – Main connectivities found in HMBC for compound 20. .................. 58
Figure 31 – 3,6-Bis(1-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (21). .......................................................... 59
Figure 32 – 3,6-Bis(1-(2-amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (22). .......................................................... 60
Figure 33 – Main connectivities found in HMBC for compound 22. ............... 61
Figure 34 – Diosmin peracetate (24). ................................................................. 62
Figure 35 – Rutin peracetate (26). ................................................................. 63
Figure 36 – Main connectivities found in HMBC for compound 26. ............... 65
Figure 37 – Naringin persulfate (27). ................................................................. 65
Figure 38 – Main connectivities found in HMBC for compound 27. ............... 67
Figure 39 – Representation of the coagulation cascade and the classical clotting assays. ......................................................................................................................... 70
Figure 40 – Dose-dependent effects of compounds 16 and 27 on APTT, PT, and TT clotting assays using human pooled plasma, expressed as ratio of clotting time in the presence/absence of compound. ................................................................. 72
Figure 41 – Dose-dependent effects of compound 22 on APTT, PT, and TT clotting assays using human pooled plasma, expressed as ratio of clotting time in the presence/absence of compound. ................................................................. 74
# TABLE INDEX

Table 1 - Advantages and disadvantages of penetration enhancers. ............................... 18
Table 2 – Tested conditions for acetylation of compound 5 ................................................ 26
Table 3 – IR data of compound 6 ...................................................................................... 41
Table 4 – IR data of compound 7 ...................................................................................... 43
Table 5 – IR data of compound 10 ..................................................................................... 44
Table 6 – IR data of compound 11 ..................................................................................... 46
Table 7 – IR data of compound 12 ..................................................................................... 48
Table 8 – IR data of compound 13 ..................................................................................... 49
Table 9 – IR data of compound 14 ..................................................................................... 50
Table 10 – IR data of compound 15 ................................................................................... 51
Table 11 – IR of compound 16 .......................................................................................... 53
Table 12 – IR data of compound 19 ................................................................................... 55
Table 13 – IR data of compound 20 ................................................................................... 57
Table 14 – IR data of compound 21 ................................................................................... 59
Table 15 - IR data of compound 22 ................................................................................... 60
Table 16 – IR data of compound 24 ................................................................................... 62
Table 17 – IR data of compound 26 ................................................................................... 64
Table 18 – IR data of compound 27 ................................................................................... 66
Table 19 – Cell growth inhibition activity displayed by compounds 6, 24, and 26 on three human tumor cell lines. ......................................................................................... 68
Table 20 – Cell growth inhibition activity of compounds 6 and 26 on three human glioblastoma cell lines. ................................................................................................. 69
Table 21 - Effects of sulfated compounds 16 and 27 on blood coagulation ...................... 73
SCHEME INDEX

Scheme 1 - Activation of DOCA..........................................................5
Scheme 2 - Direct conjugation of heparin and DOCA-NHS.....................6
Scheme 3 - Reaction of DOCA-NHS with ethylenediamine (EDA)..............7
Scheme 4 - Activation of the carboxylic acid of heparin. ......................8
Scheme 5 - Reaction of activated heparin and DOCA-NH₂. .....................9
Scheme 6 - Synthesis of carbamate DOCA. ..........................................10
Scheme 7 - Conjugation of heparin with carbamate DOCA.....................11
Scheme 8 - Introduction of a carboxyl moiety in cholesterol..................13
Scheme 9 - Synthesis of mangiferin peracetate (6)................................27
Scheme 10 - Synthesis of mangiferin heptaacetate (7)............................27
Scheme 11 - Synthesis of naringin-di-deoxycholate (10). .......................29
Scheme 12 - Synthesis of methyl 4'-naringin acetate (11). .....................30
Scheme 13 - Synthesis of 4'-naringin acetic acid (12). ..........................31
Scheme 14 - Synthesis of succinimido deoxycholate (DOCA-NHS, 13).....31
Scheme 15 - Synthesis of N-deoxycholylethylendiamine (DOCA-NH₂, 14).32
Scheme 16 - Synthesis of 4'-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15). .................................................................33

Scheme 17 - Synthesis of 4'-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide persulfate (16). .................................................................34
Scheme 18 - Synthesis of 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H,1,2,3-triazole-4-yl)methoxy)xanthone (19). .................................................................35
Scheme 19 - Synthesis of 3,6-bis(1-(2-(acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H,1,2,3-triazole-4-yl)methoxy)xanthone (20). .................................................................36
Scheme 20 - Synthesis of 3,6-bis(1-(2-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H,1,2,3-triazole-4-yl)methoxy)xanthone (21). .................................................................37
Scheme 21 - Synthesis of 3,6-bis(1-(2-(2-amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H,1,2,3-triazole-4-yl)methoxy)xanthone (22). .................................................................38
Scheme 22 - Synthesis of diosmin peracetate (24)..................................39
Scheme 23 - Synthesis of rutin peracetate (26). ....................................39
Scheme 24 - Synthesis of naringin persulfate (27)..................................40

XVII
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ABSTRACT

According to World Health Organization, cardiovascular diseases are the first cause of death worldwide. Although health improved in the last decades, lifestyle changes led to an increased incidence of cardiovascular diseases. Currently, the available antithrombotic drugs are associated with significant drawbacks that limit their use and the development of more advantageous drugs with less secondary effects is necessary. A new class of polysulfated small-molecules with anticoagulant and antiplatelet activities was discovered in LQOF. However, these polysulfated derivatives showed poor antithrombotic efficacy by in vivo oral administration in mice, predicted to be due to poor absorption in the GI tract. The main aim of this work was to improve the oral bioavailability of these compounds. In order to get new optimized analogues two strategies were considered: i) obtaining conjugates with bile acids and ii) introduction of a triazole ring.

In this dissertation sixteen compounds were synthesized, ten of which were obtained and characterized for the first time, including three sulfated derivatives.

Naringin-deoxycholic acid conjugate 15 was obtained through a crosslinking reaction using TBTU as coupling reagent. Triazole linked xanthone glycoside 21 was obtained through a copper(I)-catalyzed alkyne-azide cycloaddition following by O- and N-deacetylation. Sulfation was successfully achieved with triethylamine-sulfur trioxide adduct under microwave irradiation. Some intermediates (compounds 6, 24, and 26) were tested for cell growth inhibitory activity. Rutin peracetate (26) showed good GI_{50} on six human tumor cell lines in the micromolar range.

The three sulfated derivatives (compounds 16, 22, and 27) were screened for anticoagulant activity using the three classic clotting times APTT, PT, and TT. All the sulfated compounds prolonged the clotting times, and the most active compound was persulfated naringin-deoxycholic acid conjugate 16 exhibiting an APTT_{2} in the micromolar range (44.2 ± 0.2 µM). These new optimized analogues with anticoagulant activity are expected to cross the GI tract membranes after oral administration.

Keywords: polyphenols; naringin; anticoagulant activity; oral bioavailability
RESUMO

Segundo a Organização Mundial de Saúde as doenças cardiovasculares são a principal causa de morte em todo o mundo. Apesar de ter havido um aumento na qualidade da saúde nos últimos anos, os hábitos de vida têm-se modificado o que levou ao aumento da incidência destas doenças. Atualmente, os fármacos antitrombóticos disponíveis estão associados a desvantagens que limitam o seu uso e é necessária a descoberta de melhores fármacos com menos efeitos secundários. No LQOF foi descoberta uma nova classe de pequenas moléculas polissulfatadas com atividade anticoagulante e antiagregante plaquetária. No entanto, estas moléculas demonstraram baixa eficácia antitrombótica após administração oral em ratinhos in vivo comportamento associado a baixa absorção no trato intestinal. O principal objetivo desta dissertação foi melhorar a biodisponibilidade oral desta nova classe de pequenas moléculas polissulfatadas. Duas estratégias foram aplicadas no sentido de se atingir o objetivo proposto: i) conjugação com ácidos biliares e ii) introdução de um anel triazole.

Nesta dissertação foram sintetizados dezasseis compostos, dez dos quais foram descritos e caracterizados pela primeira vez, incluindo três compostos sulfatados.

O conjugado naringina-ácido desoxicólico 15 foi obtido através de uma reação de ligação cruzada aplicando o TBTU como reagente de acoplamento. A xantona glicosilada ligada pelo anel triazole 21 foi obtida através de uma cicloadição alcino-azida catalizada por cobre (I), seguida de O- e N-desacetilação. A sulfatação foi realizada em micro-ondas utilizando aduto de trióxido de enxofre e trietilamina como reagente de sulfatação.

Alguns intermediários acetilados (compostos 6, 24 e 26) foram testados pela sua inibição do crescimento celular, tendo-se destacado a rutina peracetilada (26) como melhor composto, apresentando IG_{50} na ordem dos micromolar.

Os compostos sulfatados obtidos (compostos 16, 22 e 27) foram testados in vitro quanto à sua atividade anticoagulante através dos testes clássicos de coagulação APTT, PT e TT. Os três compostos testados prolongaram o tempo de coagulação e o conjugado sulfatado naringina-acido desoxicólico 16 foi o mais ativo, exibindo um APTT_{2} na ordem dos micromolar (44,2 ± 0,2 µM).

É esperado que os novos análogos otimizados com atividade anticoagulante atravessem as membranas no trato gastrointestinal, após administração oral.

Palavras-chave: polifenóis; naringina; atividade anticoagulante; biodisponibilidade oral
ABBREVIATIONS

4-NPC - 4-Nitrophenyl chloroformate
APTT - Activated partial thromboplastin time
APTT₂ – Concentration required to double the activated partial thromboplastin time
brd - broad duplet
brs - broad singlet
d - duplet
DCC - N,N'-Dicyclohexylcarbodiimide
dd - double duplet
DMA - Dimethylacetamide
DMF - Dimethylformamide
DMSO - Dimethylsulfoxide
DOCA - Deoxycholic acid
DOCA-NH₂ - N-Deoxycholylethylamine
DOCA-NHS - Succinimido deoxycholate
EDA - Ethylenediamine
EDAC - 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EtOH - Ethanol
FA - Formamide
FXa - Factor Xa
GA - 18β-Glycyrrhetinic acid
GI – Gastrointestinal
GI₅₀ – concentration required to inhibit growth by 50%.
HMBC - Heteronuclear multiple bond correlation
HRMS – High resolution mass spectrometry
Potential Orally-Active Heparin-Like Compounds: Synthesis and Anticoagulant Activity

HSQC - Heteronuclear single quantum correlation
IR - Infrared
J - Coupling constant
LMWH - Low molecular weight heparins
LQOF – Laboratório de Química Orgânica e Farmacêutica
m - multiplet
MCC - Mono-N-carboxymethyl chitosan
MeOH - Methanol
MW - Microwave
NaOAc - Sodium acetate
NHS - N-Hydroxysuccinimide
NMR - Nuclear magnetic resonance
PCP-Cys - Polycarbophil-cystein
PT - Prothrombin time
Py - Pyridine
q - quartet
s - singlet
SNAC - Sodium N-(8-(2-hydroxybenzoyl) amino) caprylate
SNAD - Sodium N-(10-(2-hydroxybenzoyl) amino) decanoate
SNOCC - N-Sulfonato-N,O-carboxymethylchitosan
t - triplet
TBTU - 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoro borate
THF - Tetrahydrofuran
TLC - Thin layer chromatography
TT - Thrombin time
UFH - Unfractioned heparin
XXVI
OUTLINE OF THE DISSERTATION

The present dissertation is structured in six chapters:

INTRODUCTION

The first chapter includes a brief introduction of oral bioavailability of drugs, a state of the art concerning the strategies used to improve oral bioavailability of heparin. The aims of this dissertation are presented at the end of this chapter.

RESULTS AND DISCUSSION

In this chapter, results obtained from the synthesis, structure elucidation, and biological activity will be presented and discussed.

EXPERIMENTAL SECTION

In this chapter, the experimental procedures for the synthesis, structure characterization, and anticoagulant activity of the synthesized compounds are explained.

CONCLUSIONS

This chapter includes the main conclusions of the developed work.

REFERENCES

In this chapter, the references cited throughout the thesis are presented. The references followed the American Chemical Society style guide. The main bibliographic research motors were ISI Web of Knowledge, from Thomson Reuters, Scopus, PubMed and Google Scholar.

APPENDICES

The last chapter contains folding tables with spectroscopic data of the synthesized compounds.
CHAPTER 1 - INTRODUCTION
1.1. Oral bioavailability of drugs

In the last decades, the focus in the optimization of drug candidates has changed from improvement of potency to improvement of drug-like properties. Drug-like properties are defined as properties that drug candidates should have to become successful drug products.1 Nowadays, drug-like properties are evaluated early, in the drug discovery pipeline, in order to eliminate compounds that could fail in clinical trials.

Oral administration is the most desired administration route and thus oral bioavailability plays an important role in drug discovery and development. Poor bioavailability by oral administration affects drug’s efficacy and despite other administration routes can be chosen, this option should be avoided.

Several factors affect oral bioavailability of drugs, some being related to chemical structure of the drug (e.g. permeability, solubility) and others to the mechanisms developed by the organism to get rid of xenobiotics (e.g. first pass metabolism and efflux mechanisms).

In the gastrointestinal (GI) tract, drugs pass through biological membranes to reach the blood circulation using several mechanisms. Passive permeation is the mechanism more frequent in drug absorption and is related with solubility properties. Diverse strategies have been developed in order to surpass the poor oral absorption of drugs. Among these strategies are prodrugs, drug conjugates, structure optimisation and drug formulation.

Prodrug approach is one of the most successful strategy used to improve oral bioavailability of drugs and almost 10% of market drugs are considered prodrugs.2 International Union of Applied Chemistry defines prodrug as a “drug containing specialized nontoxic protective groups used in a transient manner to alter or to eliminate undesirable properties in the parent molecule”.3, 4 Prodrugs are inactive compounds that need to be bioactivated to have activity. Activation is achieved through modification of the drug’s structure. This activation can be enzymatic or chemical before or after absorption.

Drug conjugates are emerging as a promising strategy to improve oral bioavailability of peptides and macromolecules that usually have large molecular weight and fail to be absorbed in the GI tract. 5-7 Drug conjugates have also been applied to low molecular weight drugs.8, 9 Drug conjugates differ from prodrugs because they retain biological activity. Drugs can be conjugated with molecules which: i) will add lipophilic properties to the drug improving permeability through lipid membranes or ii) will be recognized by membrane transporters allowing the conjugate to cross the membrane through an active mechanism.

Deoxycholic acid (DOCA) and vitamin B_{12} have been used to conjugate with both large and low molecular weight drugs. DOCA has been conjugated with anticoagulants, antiviral, antifungal and antimicrobial drugs.8, 9 Vitamin B_{12} has been conjugated with antidiabetics.
and anti-obesity drugs.\textsuperscript{5, 7, 10} Drugs conjugated with these molecules will not pass the GI tract membranes through passive mechanisms as there are specific receptors to uptake bile acids and vitamins. DOCA is absorbed through apical sodium dependent bile acid transporter while vitamin B\textsubscript{12} is absorbed by receptor-mediated endocytosis at intrinsic factor–B\textsubscript{12} receptor in the apical region of enterocytes.\textsuperscript{11, 12}

Structure optimization involves molecular modifications to improve pharmacokinetic properties and to increase the oral availability of drug candidates. There are a set of rules that predict if drugs are going to reach blood circulation successfully by passive permeation. The most known are the rules of five (Lipinski rules) and three.\textsuperscript{13, 14} These rules were establish through the systematic study of properties of a great number of drugs that use passive permeation as the main mechanism to cross biological membranes in the GI tract and are widely used to predict oral availability in drug discovery and development.

Drug formulation regards to the use of excipients or micro and nanoparticles that will increase oral absorption of drugs. This strategy is applied mainly for poorly water-soluble drugs.

There are a great number of strategies to improve oral bioavailability of drugs that are applied in different stages and having different impacts on drug pharmacokinetic profiles.

1.2. Strategies to obtain orally-active heparins

Cardiovascular diseases are the leading cause of deaths in developed countries. New oral anticoagulants were recently introduced in the market, namely dabigatran (direct thrombin inhibitor), rivaroxaban, and apixaban - factor Xa (FXa) inhibitors -, but there are some hesitations about their wide use in the treatment of thromboembolic diseases.\textsuperscript{15} These new anticoagulants seem to be more advantageous than the coumarinic oral anticoagulants. Although orally-active, these drugs lack the polypharmacological actions of Heparin or unfractioned heparin (UFH) and low molecular weight heparins (LMWH) which are thought to be involved beyond the coagulation cascade\textsuperscript{16}, with antimitastic\textsuperscript{17} and anti-inflammatory activities\textsuperscript{18}.

Heparin has been used in the clinic for more than 80 years and continues to be widely used in the treatment of thromboembolic events.\textsuperscript{19} Heparin (Figure 1) is a mixture of highly sulfated glycosaminoglycans with a molecular weight between 5–30 kDa and is one of the most negative charged molecules in Nature.\textsuperscript{20-22} Heparin is an indirect inhibitor of FXa and thrombin. LMWH (Figure 1) are also mixtures of glycosaminoglycans but with mean molecular weight of about 5 kDa.\textsuperscript{23} LMWH derive from heparin by chemical or enzymatic depolymerization and have a more predictable pharmacokinetic profile.\textsuperscript{24}
Potential Orally-Active Heparin-Like Compounds: Synthesis and Anticoagulant Activity

Figure 1 – Unfractioned heparin (UFH) and low molecular weight heparins (LMWH).

Compared with warfarin, the most prescribed oral anticoagulant\textsuperscript{25}, heparins have several advantages that surpass the fact that they are administered intravenously (Figure 2).\textsuperscript{26, 27}

Heparin and LMWH

- No frequent monitoring
- Fast onset of action
- No dose adjustments
- Low food-drug and drug-drug interactions
- Suitable during pregnancy
- Other potential biological activities

Figure 2 - Advantages of heparins over other anticoagulant drugs.

LMWH can be administered subcutaneously while UFH is administered intravenously, however none exhibit oral bioavailability due to their highly negative charge, large molecular weight,\textsuperscript{28} and rapid metabolism in the GI tract (Figure 3).\textsuperscript{29}
There have been attempts to find suitable strategies that promote the absorption of heparin in the GI tract and these can be divided in three main categories: drug conjugates, formulation with penetration enhancers, and micro and nanoparticle formulations.

1.2.1. Drug conjugates

Following a drug conjugate strategy, heparin has been covalently bond to other molecules in order to achieve oral bioavailability in one of two ways: increasing lipophilicity and permeability or enabling absorption via transporter proteins or receptor mediated endocytosis.

There are some small-molecules that have been proven to be suitable choices for conjugation with heparin: DOCA and lipids.

1.2.1.1. Heparin-deoxycholic acid conjugates

DOCA (Figure 4) is a secondary bile acid produced from cholesterol and one of its functions is to stimulate the absorption of lipophilic molecules in the intestine.
DOCA is absorbed in the intestinal membrane through bile acid transporters and this mechanism of absorption would also increase the transcellular absorption of heparin. Studies proved that heparin-DOCA conjugates increased the intestinal absorption of heparin, thus increasing its oral bioavailability. Anticoagulant activity of heparin is conserved in the conjugate. DOCA is a naturally-occurring substance, so its oral administration may involve few toxic effects. In fact, Lee, et al. carried out histological examination of the membrane of the GI tissue after oral administration of heparin-DOCA, and no damage to the microvilli and the cell layer was observed.

DOCA was conjugated with heparin using several synthetic approaches, although all of them involve the formation of an amide bond that will be cleaved after absorption.

To perform direct conjugation of the carboxylic acid of DOCA with the amine groups of heparin, carboxylic acid of DOCA has to be activated (Scheme 1).

After activation, succinimido deoxycholate (DOCA-NHS) reacts instantaneously with heparin to form the conjugate (Scheme 2).
Another synthetic approach involves the coupling of the carboxylic acid of heparin and the primary amine of \textit{N}-deoxycholyethylene diamine (DOCA-NH$_2$) previously prepared. DOCA-NH$_2$ is obtained through the reaction of DOCA-NHS with ethylenediamine (EDA) (Scheme 3).
Potential Orally-Active Heparin-Like Compounds: Synthesis and Anticoagulant Activity

Scheme 3 - Reaction of DOCA-NHS with ethylenediamine (EDA). DMF - dimethylformamide; r.t. - room temperature.

Activation of the carboxylic acid of heparin is performed before the conjugation reaction (Scheme 4). Generally, activating reagents used are 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) alone\(^6\)\(^{36-39}\) or in combination with \(N\)-hydroxysuccinimide (NHS)\(^40\).
Scheme 4 - Activation of the carboxylic acid of heparin. EDAC - 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; NHS - N-hydroxysuccinimide; FA – formamide; DMF – dimethylformamide.

Activated heparin and DOCA-NH₂ react instantaneously as shown in Scheme 5 to originate the heparin-DOCA conjugate.
Conjugation heparin with DOCA through the modification of an aliphatic hydroxyl group of DOCA was also described.\textsuperscript{41, 42} This strategy consists in increasing the reactivity of 3-OH with the introduction of 4-nitrophenyl chloroformate (4-NPC) following reaction 4-methylmorpholine and with EDA as shown in \textbf{Scheme 6}. 

\textbf{Scheme 5} - Reaction of activated heparin and DOCA-NH\textsubscript{2}. FA - formamide; DMF - dimethylformamide; r.t. - room temperature.
Scheme 6 - Synthesis of carbamate DOCA. 4-NPC - 4-nitrophenyl chloroformate; NMM - 4-methylmorpholine; EDA - ethylenediamine.

Carbamate DOCA further reacts with activated heparin as shown in Scheme 7.
1.2.1.2. Heparin-fatty acids and heparin-cholesterol conjugates

Fatty acids are carboxylic acids with a more or less long aliphatic chain, which make them amphiphilic compounds. They can be attached to other compounds, making them more lipophilic which, in the case of heparin, is useful in the attempt to enhance its absorption in the GI tract.

Lee et al. prepared fatty acids-heparin conjugates with palmitic acid and lauric acid (Figure 5). The conjugates proved to retain anticoagulant activity and increased the absorption of heparin in rats, through the increase of lipophilicity. Palmitic acid and lauric acid were coupled with amine groups of heparin through their carboxylic groups. The strategy is the same used for heparin-DOCA conjugates.

Paliwal et al. also synthesized fatty acids-heparin conjugates, with stearic acid, palmitic acid and myristic acid (Figure 5), coupling the carboxylic group of lipids with the amine groups of heparin. As Lee et al., they proved that these conjugates retained anticoagulant activity.
Additionally, heparin was conjugated with cholesterol, a sterol that is present and plays an essential role in animal cell membranes. Nevertheless, heparin-DOCA conjugates showed higher prolonging effects than heparin-cholesterol conjugates.\textsuperscript{35}

For the heparin–cholesterol conjugate, most of the experimental procedures were similar to those with heparin-DOCA. Amide coupling was preceded by the introduction of a carboxyl group. This was achieved by alkylation of the hydroxyl group, in which cholesterol reacted with chloroacetic acid (Scheme 8).\textsuperscript{35}
Potential Orally-Active Heparin-Like Compounds: Synthesis and Anticoagulant Activity

Scheme 8 - Introduction of a carboxyl moiety in cholesterol.

In 2000, a study showed that heparin-lipid conjugates, such as heparin-cholesterol, heparin-palmitic acid, and heparin-lauric acid conjugates had a lower absorption in the GI tract, compared with heparin-DOCA conjugate. These findings suggest that DOCA increases heparin absorption in the GI tract through receptor-mediated uptake.

1.2.2. Formulations with penetration enhancers

Penetration enhancers are compounds used to improve absorption of drugs in the GI tract either by opening the tight junctions between adjacent cells, enabling paracellular absorption - permeation enhancers - or by increasing the lipophilic properties of the drug enabling transcellular absorption - absorption enhancers (Figure 6).

Figure 6 – Transcellular and paracellular absorption in the GI tract (adapted from 45).
The weak association between enhancers and drugs allows the spontaneous release of the drug into the circulation.\textsuperscript{46} However, many of the compounds examined \textit{in vitro} as membrane permeation enhancers cause cytotoxicity or membrane damage. In addition, systemic toxic side effects of these compounds cannot be excluded.\textsuperscript{47} Absorption enhancers are low molecular weight compounds that in contrast to permeation enhancers do not compromise the integrity of the intestinal epithelium.\textsuperscript{48} In this section, some penetration enhancers that have already been used with heparin and LMWH will be revised.

\subsection*{1.2.2.1. Permeation enhancers}

- \textit{Sodium caprate}

Fatty acids can also be applied as permeation enhancers, enhancing the paracellular absorption of hydrophilic drugs at millimolar concentrations. Sodium caprate (\textit{Figure 7}), the sodium salt of the aliphatic saturated 10-carbon fatty acid, capric acid, is the most comprehensively characterized medium length fatty acid as permeation enhancer.\textsuperscript{46, 48} Oral delivery of LMWH using sodium caprate as penetration enhancer reached therapeutic levels and its cytotoxicity in Caco-2 cells was not found to be severe.\textsuperscript{49}

\begin{center}
\textbf{Figure 7} - Sodium caprate.
\end{center}

- \textit{Glycyrrhetinic acid}

18β-Glycyrrhetinic acid (GA) (\textit{Figure 8}) is a pentacyclic triterpenoid amyrin derivative obtained from the hydrolysis of glycyrrhizic acid, naturally present in the roots of the plant \textit{Glycyrrhiza glabra}. GA was tested as a penetration enhancer in order to increase the intestinal absorption of LMWH by Motlekar \textit{et al.}.\textsuperscript{50} Absorption of a LMWH was increased both \textit{in vitro} and \textit{in vivo} after co-administration with GA. After exposure to GA, no significant toxicity in Caco-2 cells monolayers was found, at relatively low concentrations. The authors hypothesised that GA could possibly be used as a penetration enhancer through paracellular absorption.\textsuperscript{51} Although the study was conducted by Hisamitsu Pharmaceutical Co., Inc., the company does not seems to be developing a formulation of oral heparin with GA at the moment, being GA applied only in transdermal formulations.\textsuperscript{52}
Recently, anticoagulant properties through uncompetitive inhibition of FXa were described for GA.$^{53}$

- **Mucoadhesive polymers**

Mucoadhesive polymers are polymers that have the capacity to adhere to mucous membranes. Chitosan derivatives and thiolated polymers will be addressed in this subsection as they increased the absorption of heparin or LMWH in the GI tract.

Chitosan is a polysaccharide with mucoadhesive properties that comprises glucosamine and N-acetylglucosamine subunits.$^{54}$ *In vitro* studies have shown that chitosan opens epithelial tight junctions in a concentration- and pH-dependent way.$^{54}$ However, chitosan was incompatible with LMWH$^{55}$ and chitosan derivatives were prepared.$^{56,57}$

Thanou *et al.* synthesized mono-N-carboxymethyl chitosan (MCC) (*Figure 9*), a polyampholyte chitosan derivative that has shown to increase paracellular absorption of LMWH *in vitro*, in Caco-2 cells monolayers.$^{56}$ MCC also increased the intestinal absorption of LMWH *in vivo* in rats. When the complex passes the intestinal barrier, it dissociates to yield the active macromolecule.

A sulfonate derivative of N,O-carboxymethyl chitosan (SNOCC) (*Figure 9*) increased the permeation and absorption of LMWH both *in vitro* in Caco-2 cells monolayer and *in vivo* in rats through intraduodenal administration.$^{57}$ SNOCC compared with others absorption enhancers has the advantage of not being absorbed in the GI tract, which is due to its high molecular weight.

Thiolated polymers or thiomers have improved mucoadhesion properties and permeation enhancing properties due to the thiol groups.$^{56}$ The presence of thiol groups offer the advantage to form disulfide bonds between these novel polymers and the mucus gel layer, which mimics natural mechanism of secreted mucus glycoproteins, which are also covalently anchored in the mucus layer by the formation of disulfide bonds.$^{59}$ Polycarbophil-cysteine (PCP-Cys) (*Figure 9*) is a thiomer that was found to increase the absorption of
LMWH to anticoagulant levels.\textsuperscript{58} The oral administration of heparin with PCP–Cys resulted in a significantly increased absorption of LMWH compared with control tablets comprising unmodified PCP or to an orally given aqueous heparin solution.\textsuperscript{58} Figure 9 - Mono-\textit{N}\textendash carboxymethyl chitosan (MCC), a sulfonate derivative of \textit{N,\textit{O}}-carboxymethyl chitosan (SNOCC), and thiolated polycarbophil (PCP-Cys).

- **SNAC and SNAD**

  Sodium \textit{N}-\{8 [2-hydroxybenzoyl] amino\} caprylate (SNAD) and sodium \textit{N}-\{10-(2-hydroxybenzoyl) amino\} decanoate (SNAC) (Figure 10) interact non-covalently with heparin, neutralizing its negative ionic charge to render it more lipophilic.\textsuperscript{44} SNAC increase the absorption of heparin through the GI tract in therapeutic doses.\textsuperscript{60} Once the complex crosses the membrane through a paracellular route, SNAC dissociates from the therapeutic agent.\textsuperscript{46, 61} Figure 10 - Sodium \textit{N}-\{8 [2-hydroxybenzoyl] amino\} caprylate (SNAC) and sodium \textit{N}-\{10-(2-hydroxybenzoyl) amino\} decanoate (SNAD).

  Oral heparin/SNAC entered clinical trials and showed good results in healthy volunteers and in patients undergoing elective total hip arthroplasty.\textsuperscript{62} LMWH/SNAD has also shown to prevent deep venous thrombosis after oral route.\textsuperscript{63} These studies demonstrated for the first time that heparins can be effectively orally delivered into the bloodstream in patients.\textsuperscript{64} However, there are still no formulations with orally-active heparin currently in the market.
1.2.2.2. Absorption enhancers

- **Polycationic lipophilic-core dendrons**

Polycationic lipophilic-core dendrons (Figure 11) are absorption enhancers that have proven to enhance absorption of LMWH in rats. They form lipophilic ion-pairs with the polyanionic LMWH making them hydrophobic. This ion-pair model of absorption assumes that the dendrons are absorbed as a complex with LMWH. However, poor aqueous solubility of the complex dendron-LMWH limited its absorption.

![Polycationic lipophilic-core dendrons](image)

**Figure 11** - Polycationic lipophilic-core dendrons.

- **N-Deoxycholyethylene diamine (DOCA-NH₂)**

Through lipophilic ion pairing, Lee, *et al.* designed a DOCA derivative, DOCA-NH₂, (see Introduction, Scheme 3) to complex with LMWH. The complex was dissolved in propylene glycol and administered to rats by oral gavage. The physically association of DOCA-NH₂ with LMWH turn the later more lipophilic and *in vivo* experiments indicated that DOCA-NH₂ significantly affected the oral absorption of the LMWH, and at the molar ratio of 1:5 the oral absorption of LMWH was high. Although, the complex LMWH/DOCA-NH₂ has reduced solubility due to the larger size of the complex and propylene glycol had to be used as solubilizer. The oral absorption of LMWH/DOCA-NH₂ complex was higher than those of LMWH/DOCA complex. The mechanism used to improve LMWH absorption is not clear and may be by bile acids transporters or passive absorption. However, no toxicological studies were performed and side effects cannot be excluded.

The use of penetration enhancers appears to be a good strategy to improve oral availability of heparin. An advantage is the preservation of the chemical structure of the drug. However, toxic effects must be evaluated. Advantages and disadvantages of the penetration enhancers presented are illustrated in Table 1.
### Table 1 - Advantages and disadvantages of penetration enhancers.

<table>
<thead>
<tr>
<th>Penetration enhancer</th>
<th>Mechanism</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium caprate*</td>
<td>Paracellular absorption</td>
<td>No severe toxicity in Caco-2 cells; increase absorption at millimolar range</td>
<td>Local toxicity</td>
</tr>
<tr>
<td>GA*</td>
<td>Paracellular absorption</td>
<td>No significant toxicity in Caco-2 cells monolayers after exposure to GA 50</td>
<td>Not described</td>
</tr>
<tr>
<td>Chitosan derivatives (SNOCC and MCC)*</td>
<td>Increase the residence time in the GI tract and promote paracellular absorption of the drug</td>
<td>Biocompatible; not absorbed (elevated molecular weight) - few systemic side/toxic effects</td>
<td>Lower efficiency of MCC when compared with other delivery agents</td>
</tr>
<tr>
<td>Thiomers*</td>
<td>Paracellular absorption</td>
<td>Improved mucoadhesion properties and permeation enhancing properties due to the thiol groups</td>
<td>Possible toxic effects</td>
</tr>
<tr>
<td>SNAC and SNAD*</td>
<td>Paracellular absorption</td>
<td>Release the drug after absorption</td>
<td>Toxic effects (absorbed with the drug)</td>
</tr>
<tr>
<td>Polycationic lipophilic-core dendrons#</td>
<td>Transcellular absorption</td>
<td>Suitable for ionizable molecules</td>
<td>Absorbed with the drug (toxic effects); poor aqueous solubility of the complex dendron-LMWH</td>
</tr>
<tr>
<td>DOCA-NH₂#</td>
<td>Transcellular absorption</td>
<td>Recognition through bile acid transporters</td>
<td>Not described</td>
</tr>
</tbody>
</table>

*permeation enhancer; #absorption enhancer.
1.2.3. **Micro and nanotechnology**

Micro and nanotechnology constitute two approaches widely used in drug delivery to enhance drug’s pharmacokinetic profile and decrease its side effects.

Both polymeric micro and nanoparticles have been used to improve oral absorption of heparin. Biodegradable polymers (poly-\(\varepsilon\)-caprolactone and poly-D,L-lactic-co-glycolic acid) and non-biodegradable polymers (Euparin dragit\(^\text{®}\) RS and RL) were used to prepare heparin-loaded nanoparticles.\(^{68, 69, 71, 72}\) It was observed that heparin maintained its anticoagulation activity, yet only *in vitro* studies were performed. These polymers were also used to prepare nanoparticles to encapsulate heparin.\(^{70}\) Initial *in vitro* tests showed satisfactory encapsulation efficiency and controlled drug release with retention of the anticoagulant activity.\(^{72}\) Further *in vivo* studies in rabbits showed oral absorption of heparin after administration.\(^{68}\)

Later, LMWH (tinzaparin)-loaded nanoparticles were prepared using polyester and polycationic polymethacrylate.\(^{71}\) Oral absorption of heparin was improved and its anticoagulant effect was prolonged for up to 8 hours.

It can be found in the literature a combination of strategies through the incorporation of lipid-heparin conjugates in solid lipid nanoparticles.\(^{43}\) Incorporation of these conjugates into solid lipid nanoparticles significantly improved the bioavailability of LMWH after oral route administration with insignificant toxicity to different GI tissue.

Nevertheless, this approach has drawbacks that are related with the delay they create in drug absorption and the lack of control retained over absorption time as a result of the variability in intestinal motility and gastric emptying. Toxicity also has to be considered, as long as polymeric materials modify tight junctions and could lead to the absorption of endotoxins and other potentially toxic compounds.\(^{73}\)
1.3. **Aims: development of polysulfated small-molecules towards new oral antithrombotic agents**

In order to discover new antithrombotic drugs with better pharmacokinetic profiles one strategy was adopted in Laboratório de Química Orgânica e Farmacêutica (LQOF): incorporate an oligosulfated moiety into a phenolic molecule (flavonoid and xanthone) in order to mimetize anticoagulant polysaccharides (Figure 12) while increasing the overall hydrophobicity.\(^{74, 75}\) In contrast to UFH, polysulfated small-molecules have less charge density, reduced anionic character, higher hydrophobic nature, and have a defined composition and feasible synthesis.

![Chemical structures](image)

**Figure 12** – Examples of polysulfated glycosidic flavonoids/xanthonoids: rutin persulfate (1), 3,6-\((O-\beta\text{-glucopyranosyl})\) xanthone persulfate (2), mangiferin heptasulfate (3) diosmin persulfate (4).

The anticoagulant activity of these sulfated compounds such as exemplified in Figure 12 (compounds 1-4) was evaluated *in vitro* by the classical clotting times. Polysulfated compounds 1-4 prolonged activated partial thromboplastin time (APTT)\(^{74, 75}\) and the most potent compound (compound 2) exhibited an APPT\(_2\) (concentration required to double the activated partial thromboplastin time) in the micromolar range (ca 60 \(\mu\text{M}\)).\(^{75}\) The prothrombin time (PT) or thrombin time (TT) pathways were less affected. After intraperitoneal administration in mice, polysulfated compounds revealed a systemic anticoagulant action with a rapid onset of action.\(^{74, 75}\) Polysulfated small-molecules showed high solubility, stability in human plasma, and efficacy as antithrombotic, and preliminarily
Potential Orally-Active Heparin-Like Compounds: Synthesis and Anticoagulant Activity

*in vivo* toxicological studies in mice showed that these small-molecules are not expected to induce acute hepatic toxicity.\(^{74, 75}\)

Furthermore, some of the synthesized polysulfated derivatives exhibited simultaneously anticoagulant and antiplatelet activities.\(^{76}\) Even though a combined anticoagulant and antiplatelet therapy for patients with multiple disorders is still discussed, compounds combining in the same molecule anticoagulant and antiplatelet activities are believed to be promising drugs in preventing and/or treat both venous and arterial thrombosis. Dual inhibitors should be advantageous, due to expected less complex pharmacokinetics, lower incidence of side effects, and less demanding clinical studies.\(^{77}\)

Nevertheless, after oral administration, none of the tested compounds was active.\(^{78}\)

Thus, the main purpose of this dissertation was the improvement of oral bioavailability of these polysulfated small-molecules.

Two strategies were considered to achieve this purpose:

i) **BILE ACID CONJUGATION**

Conjugation of DOCA was chosen as a potential strategy to improve oral bioavailability of polysulfated small-molecules. DOCA is absorbed in the GI tract through specific membrane receptors and was successfully used to improve heparin bioavailability without significant toxicity (Section 1.2), in contrast to permeation enhancers. In fact, targeting membrane receptors is a strategy used for the oral delivery of drugs.\(^{31}\) Fluconazole and acyclovir have already been successfully conjugated with molecules that are recognized by intestinal receptors to increase oral bioavailability.\(^{8, 9}\)

ii) **INTRODUCTION OF A TRIAZOLE**

Introduction of a triazole in the polysulfated glycosidic small-molecules was planned as a strategy to increase lipophilicity. Triazole has been used as a strategy to improve lipophilicity of several types of drugs,\(^{79-81}\) as antifungal and anti-human immunodeficiency virus drugs.\(^{80, 81}\)

Thus, the specific aims were:

i) to synthesize new phenolic bile acid conjugates,

ii) to synthesize triazole-linked phenolic glucosides,

iii) to synthesize sulfated derivatives of the compounds referred in i and ii,

iv) to characterize through spectroscopic techniques the structure of the synthesized derivatives,
v) to screen the biological activities of the obtained compounds (i, ii, and iii).
CHAPTER 2 - RESULTS AND DISCUSSION
2.1. SYNTHESIS

2.1.1. Conjugation of mangiferin with deoxycholic acid

Mangiferin (5, Figure 13) is a naturally-occurring xanthone-\(C\)-glucoside found mainly in mango tree \textit{Mangifera indica}. Compound 5 was the first xanthone to be investigated pharmacologically\textsuperscript{82} and shows several biological activities, such as antioxidant\textsuperscript{83-85}, antidiabetic\textsuperscript{86, 87}, and antiviral.\textsuperscript{88, 89} Derivatives of compound 5 such as acetyl\textsuperscript{83, 90}, propionyl\textsuperscript{80, 91}, butyryl\textsuperscript{86}, cinnamoyl,\textsuperscript{83} benzyl\textsuperscript{92, 93}, and substituted anilyl derivatives\textsuperscript{94-96} also displaying a wide range of biological activities were described.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{mangiferin.png}
\caption{Mangiferin (5).}
\end{figure}

In LQOF, mangiferin heptasulfate (3, Figure 12) was synthesized and in \textit{in vitro} studies showed anticoagulant activity.\textsuperscript{75} However, in \textit{in vivo} studies mangiferin heptasulfate (3) was not active after oral administration.\textsuperscript{75} Permeability studies using Ussing chamber verified that the compound was not able to cross the intestinal membrane (unpublished results).

Taking these results in account, conjugation with DOCA was planned in order to increase intestinal absorption.

2.1.1.1. Acetylation of mangiferin (5)

Compound 5 has four phenolic groups with the following reactivity order: 3, 6, and 7-OH > 1-OH.\textsuperscript{97} So, it was planned to protect the phenolic groups of mangiferin except 1-OH, to be available for further conjugation with DOCA.

Acetylation is a commonly used reaction to protect functional groups, because of simple deprotection and stability of the groups in mild basic or acid conditions. To obtain the acetylated derivative of compound 5 with only 1-OH free, several conditions were attempt (Table 2).
Table 2 – Tested conditions for acetylation of compound 5.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ac₂O, Py, CH₂Cl₂, r.t.⁹⁸</td>
<td>Several partially acetylated products were obtained.</td>
</tr>
<tr>
<td>2</td>
<td>Ac₂O/AcOH, NaOAc, reflux⁹⁹</td>
<td>Several partially acetylated products were obtained.</td>
</tr>
<tr>
<td>3</td>
<td>Ac₂O, r.t.</td>
<td>No product formed</td>
</tr>
<tr>
<td>4</td>
<td>Ac₂O, NaF, MW, reflux¹⁰⁰</td>
<td>Several partially acetylated products were obtained.</td>
</tr>
<tr>
<td>5</td>
<td>Ac₂O, I₂, MW, reflux¹⁰¹</td>
<td>Mangiferin peracetate was obtained.</td>
</tr>
<tr>
<td>6</td>
<td>Ac₂O, reflux¹⁰²</td>
<td>Two principal products were obtained: mangiferin hepta- and peracetate</td>
</tr>
</tbody>
</table>

Acetic anhydride was the selected reagent to introduce acetyl groups due to its wide use in acetylation.²¹,¹⁰¹,¹⁰³-¹⁰⁵

Since acetylation can be catalysed by bases, acids, metallic and non-metallic Lewis acids, pyridine was firstly chosen as the nucleophilic catalyst (Table 2, entry 1). Nonetheless, the reaction resulted in several partially acetylated products (Table 2, entry 1).

Other bases are employed in acetylation as catalysts, such as NaOAc (sodium acetate).¹⁰³ Thus, NaOAc·3H₂O in a mixture of acetic anhydride and acetic acid was investigated (Table 2, entry 2). These conditions were unsuccessful providing also a complex mixture of partially acetylated products.

NaF, an inorganic salt, was found to be an efficient catalyst in acetylation of hydroxyl groups.¹⁰⁰ Ready availability and low cost are some advantages of NaF. When applied to mangiferin (5) in solvent-free conditions and under microwave (MW) irradiation, several partially acetylated products were also obtained (Table 2, entry 4).

Molecular iodine is described as a strong and efficient catalyst for acetylation of alcohols, amines and phenols, being an inexpensive, non-toxic, non-metallic and readily available catalyst.¹⁰⁸,¹⁰⁹ When iodine was used as catalyst in the acetylation of mangiferin (5) in solvent-free conditions using an excess of acetic anhydride under MW irradiation (Table 2, entry 5; Scheme 9), this reaction condition gave one major product, mangiferin peracetate (6) in 78% yield.
Scheme 9 - Synthesis of mangiferin peracetate (6). Ac₂O - acetic anhydride; MW - microwave.

The derivative of compound 5 with the free 1-OH was only obtained after reflux with an excess of acetic anhydride (Table 2, entry 6; Scheme 10) in 11% yield. Two products were obtained and only mangiferin heptaacetate (7) was isolated through flash chromatography column. Although the presence was detected by thin layer chromatography (TLC) by comparing with a standard, mangiferin peracetate (6) was not isolated.

Scheme 10 - Synthesis of mangiferin heptaacetate (7). Ac₂O – acetic anhydride.

2.1.1.2. Carboxymethoxymethylation of mangiferin (5)

The next synthetic step was the introduction of a carboxylic moiety at 1-position of compound 7 to increase reactivity for the following conjugation with DOCA.

Several conditions were attempt with compound 7 but all of them originated a complex mixture of compounds. A probably explanation for the formation of a mixture of products lies in the fact that acetyl groups may be replaced by alkyl groups. Due to this, conjugation with DOCA was planned for another compound: naringin.
2.1.2. Conjugation of naringin with deoxycholic acid (I)

Naringin (8, Figure 14) is a flavanone glycoside naturally present in grapefruit and other citrus fruits. Similar to other natural flavonoids, compound 8 has a wide variety of biological activities and is commercialized as an antioxidant supplement.\textsuperscript{112-114} Supplementation with compound 8 extracted from citrus fruits has been proved to decrease plasma lipid concentrations and to improve the antioxidant mechanism system.\textsuperscript{115,116} Thus, naringin (8) is a suitable model to plan potential antithrombotic derivatives.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{naringin}
\caption{Naringin (8).}
\end{figure}

Compound 8 was selected to conjugate with DOCA due to the well-established differences in the reactivity of the two phenolic groups present at 5 and 4’ positions: 4’-OH is more reactive, while 5-OH is less available due to a hydrogen interaction with the C=O at C-4.

Due to the presence of a single highly reactive phenolic group (4’-OH), direct conjugation of compound 8 with DOCA (9) was firstly attempt.

Esterification of the carboxylic acid of DOCA (9) with the 4’-OH phenolic group of compound 8 was investigated using 2-(1\textsubscript{H}-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoro borate (TBTU) as the coupling reagent (Scheme 11). Esterification of carboxylic acids with phenols is easily accessible using TBTU in the presence of triethylamine (TEA), in smooth conditions and good yields.\textsuperscript{117}

The coupling reaction is depicted in Scheme 11. Three products were detected but only compound 10 was isolated by flash chromatography column following preparative TLC in 2.2% yield. Spectroscopic data did not allow to confirm the exactly position of the second molecule of DOCA. The other two compounds were hypothesized to be monoconjugates of compound 8 with DOCA.
Scheme 11 - Synthesis of naringin-di-deoxycholate (10). TBTU - 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoro borate; TEA – triethylamine; THF – tetrahydrofuran; r.t. - room temperature.
2.1.3. Conjugation of naringin with deoxycholic acid (II)

2.1.3.1. Carbomethoxymethylation of naringin (8)

Due to the results obtained in the direct conjugation of naringin (8) with DOCA (9), carbomethoxymethylation of 4′-OH was performed in order to increase reactivity in this position.

Methyl 4′-naringin acetate (11) was obtained within 32 hours in 18% yield (Scheme 12).

![Scheme 12 - Synthesis of methyl 4′-naringin acetate (11). DMF - dimethylformamide; r.t. - room temperature.](image)

2.1.3.2. Deacetylation of methyl 4′-naringin acetate (11)

4′-Naringin acetic acid (12) was obtained using a Zemplén deacetylation18 with sodium methoxide in methanol (MeOH) at room temperature (Scheme 13). Zemplén deacetylation was selected because of the use of catalytic amounts of base (sodium methoxide), short reaction times, and excellent yields.18

After the completion of the reaction neutralization using an ion exchange column was carried out to afford compound 12 in quantitative yields.
Potential Orally-Active Heparin-Like Compounds: Synthesis and Anticoagulant Activity

**Scheme 13** - Synthesis of 4'-naringin acetic acid (12). MeONa - sodium methoxide; MeOH - methanol; r.t. - room temperature.

2.1.3.3. Synthesis of succinimido deoxycholate (13)

Before the conjugation of compound 12 with DOCA (9), activation of compound 9 was performed. Succinimido deoxycholate (DOCA-NHS, 13) was synthesized in accordance with a method previously reported for the synthesis of heparin-DOCA conjugates ([Scheme 14](#)).\(^{39}\) \(N,N\)-Dicyclohexylcarbodiimide (DCC) was used as the coupling reagent. DCC reacts firstly with the carboxylic acid of deoxycholic acid and is replaced by NHS to form the activated ester DOCA-NHS 13, which is stable and can be isolated.

**Scheme 14** - Synthesis of succinimido deoxycholate (DOCA-NHS, 13). NHS - \(N\)-hydroxysuccinimide; DCC - \(N,N\)-dicyclohexylcarbodiimide; THF - tetrahydrofuran; r.t. - room temperature.
Purification was accomplished firstly by filtration of the by-product dicyclohexylurea and then by precipitation of the product from the reaction mixture. DOCA-NHS (13) was obtained in 88% yield.

2.1.3.4. Synthesis of N-deoxycholylethylenediamine (14)

The method employed to synthesize N-deoxycholylethylenediamine (DOCA-NH$_2$, 15) was previously described in the synthesis of heparin-DOCA conjugates (Scheme 15).  

![Scheme 15](image)

**Scheme 15** - Synthesis of N-deoxycholylethylenediamine (DOCA-NH$_2$, 14). EDA - ethylenediamine; DMF - dimethylformamide; r.t. - room temperature.

The product was obtained with 81% yield in 24 hours after pouring the reaction in ice following filtration, which allowed eliminating the unreacted EDA.

2.1.3.5. Synthesis of 4'-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15)

A mixture of dimethylformamide (DMF) and H$_2$O is used as solvent in the synthesis of heparin-DOCA conjugates.  

![Scheme 16](image)

**Scheme 16** - Synthesis of 4'-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15). EDAC - 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride;  N,N'-dicyclohexylcarbodiimide: H$_2$O - water; TEA - triethylamine; DMF - dimethylformamide; TBTU - 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride;  r.t. - room temperature.
Scheme 16 - Synthesis of 4'-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15). TBTU - 2-((1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA - triethylamine; DMF - dimethylformamide; r.t. - room temperature.

The reaction occurred within 48 hours with 73% yield. After pouring the reaction in ice the product was easily isolated by filtration.

2.1.3.6. Sulfation of 4'-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15)

Sulfation of compound 15 was accomplished with MW-assisted sulfation using TEA-sulfur trioxide adduct (SO₃:TEA) in dimethylacetamide (DMA) for 2 hours at 100 °C in 50% yield (Scheme 17).

Sulfur trioxide adducts are successfully applied in the polysulfation and persulfation of polyhydroxyl molecules and are mild reagents when comparing with other sulfation reagents.\(^\text{120}\) The use of SO₃:TEA was selected due to successful application in the sulfation of alcohols in carbohydrate scaffolds and sulfation of phenols.\(^\text{121}\) MW-assisted sulfation
overcomes some limitations of the conventional methods such as long times of reaction and the use of high amount of sulfation adduct.\textsuperscript{122}

\textbf{Scheme 17} - Synthesis of 4’-naringin (\textit{N-(2-deoxycholan-24-amidoethyl)})acetamide persulfate (16). SO\textsubscript{3}:TEA - triethylamine-sulfur-trioxide adduct; DMA - dimethylacetamide; MW - microwave.

After the completion of the reaction the TEA salt of the sulfated conjugate was converted into the sodium salt with an aqueous solution of NaOAc, followed by insolubilization in ethanol (EtOH) to isolate the desirable product 16 in 50% yield.
2.1.4. Glycosylation of 3,6-dihydroxy xanthone through a triazole

2.1.4.1. Copper(I)-catalyzed alkyne-azide cycloaddition

Among the methods available to form a triazole ring, copper(I)-catalyzed alkyne-azole 1,4-cycloaddition was selected due to several advantages.\(^{123}\) 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H,1,2,3-triazole-4-yl)methoxy)xanthone (19) was synthesized reacting 3,6-bis(prop-2-yn-1-yl)oxy)-9H-xanthen-9-one (17, previously synthesized in LQOF) with 2-azidoethyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (18) (**Scheme 18**). After 30 minutes at 70ºC under MW irradiation, compound 19 was easily purified by liquid-liquid extraction and obtained in 73% yield.

![Scheme 18](image)

**Scheme 18** - Synthesis of 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H,1,2,3-triazole-4-yl)methoxy)xanthone (19). THF – tetrahydrofuran; MW - microwave.

2.1.4.2. Deacetylation of 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H,1,2,3-triazole-4-yl)methoxy)xanthone (19)

Deprotection of the glycosidic moiety of compound 19 was achieved under Zemplén conditions\(^{123}\) (**Scheme 19**) in 86% yield. The reaction was complete after 3 hours and 3,6-bis(1-(2-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H,1,2,3-triazole-4-yl)methoxy)xanthone (20) was easily isolated through filtration. Spectroscopic data
revealed that the acetyl group of the amide was not removed using these reaction conditions.

**Scheme 19** - Synthesis of 3,6-bis(1-(2-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (20). MeONa - sodium methoxide; MeOH - methanol; r.t. - room temperature.

2.1.4.3. *N*-deacetylation of 3,6-bis(1-(2-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (20)

Hydrolysis of amide bonds is achieved generally using harsh conditions, that is, strong acid or base at high temperatures, which could affect other sensitive groups in the molecule. The presence of electronegative groups in carbon or nitrogen accelerates basic catalysis, while alkyl groups in nitrogen retard both basic and acid catalysis.

Some synthetic conditions were attempt for deacetylation of *O*- and *N*-acetyl groups of compound 20. Firstly, a recent method that use mild conditions - Schwartz' reagent (bis(cyclopentadienyl)zirconium(IV) chloride hydride) - was applied, as an alternative to acid or base-catalysed hydrolysis. However, during the reaction the starting material 20 did not react probably because of low solubility.

A MW-assisted deacetylation method using ammonium salt and EDA was also attempt. Although this method was successfully applied to a wide range of amides, a complex mixture of compounds was obtained.

*N*-deacetylation was only achieved using a classic base-catalysed hydrolysis, with an aqueous solution of NaOH 20% at 100°C (Scheme 20) after hydrolysis of the *O*-acetyl group.
groups using sodium methoxide. Purification was carried out through dialysis following by filtration to furnish compound 21 in 36% yield.

Scheme 20 - Synthesis of 3,6-bis(1-(2-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (21).

2.1.4.4. Sulfation of 3,6-bis(1-(2-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (21)

Sulfation of compound 21 was achieved under MW irradiation with SO$_3$:TEA in 2 hours with 78% yield after isolation by a similar procedure as compound 16 (Scheme 21).
2.1.5. Others

In the course of this dissertation, to establish future structure-activity relationships with the final sulfated products for anticoagulant activity or with acetylated intermediates for antitumor activity other phenolic derivatives were obtained and characterized.

2.1.5.1. Acetylation of diosmin (23)

Diosmin (23) was acetylated under MW irradiation using NaF as catalyst to give diosmin peracetate (24) (Scheme 22). In contrast to mangiferin (5), acetylation of compound 23 using these reaction conditions lead to the formation of one major product which was isolated through crystallization from MeOH/H2O in 63% yield.
2.1.5.2. Acetylation of rutin (25)

Rutin peracetate (26) was obtained through the reaction of rutin (25) with acetic anhydride under conventional heating, at 130ºC, with 73% yield (Scheme 23). After completion of the reaction the product was isolated through liquid-liquid extraction following by insolubilization.

Scheme 23 - Synthesis of rutin peracetate (26). Ac₂O - acetic anhydride.
2.1.5.3. Sulfation of naringin (8)

Naringin persulfate (27) was obtained through the reaction of compound 8 with SO$_3$:TEA, under MW irradiation for 1 hour (Scheme 24).

![Scheme 24 - Synthesis of naringin persulfate (27). SO$_3$:TEA - triethylamine-sulfur trioxide adduct; DMA - dimethylacetamide; MW - microwave.](image)

Compound 27 was obtained in 48% yield after purification from inorganic salts through dialysis.
2.2. STRUCTURE ELUCIDATION

2.2.1. Mangiferin peracetate (6)

Structure elucidation of mangiferin peracetate (6, Figure 15) was established by infrared (IR) and nuclear magnetic resonance (NMR) (\(^1\)H and \(^{13}\)C) and is in accordance with those reported.\(^{99,128}\)

![Figure 15 – Mangiferin peracetate (6).](image)

IR spectrum showed two strong bands at 1754 and 1781 cm\(^{-1}\) typical of C=O ester stretching vibration which suggested the presence of acetyl groups (Table 3).

<table>
<thead>
<tr>
<th>Groups</th>
<th>(\nu) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-H aliphatic</td>
<td>2937</td>
</tr>
<tr>
<td>C=O ester</td>
<td>1781, 1754</td>
</tr>
<tr>
<td>C=O ketone</td>
<td>1664</td>
</tr>
<tr>
<td>C=C aromatic</td>
<td>1618, 1459</td>
</tr>
<tr>
<td>C-O</td>
<td>1172</td>
</tr>
</tbody>
</table>

\(\nu\) - wavenumber

\(^1\)H and \(^{13}\)C NMR data for compound 6 are presented in APPENDIX I.

Characteristic signals of the aromatic protons H-4, H-5, and H-8 appeared as singlet at \(\delta_H\) 7.25, 7.39, and 8.00 ppm, respectively, whereas the corresponding signals of these protons appeared at \(\delta_H\) 6.38, 6.87, and 7.38 ppm in the non-acetylated parent compound, mangiferin (5, also in APPENDIX I). Seven signals characteristic of aliphatic protons (the sugar moiety) were observed between \(\delta_H\) 3.80-5.73 ppm, whereas the corresponding
signals of these protons appeared between $\delta_H$ 3.09-4.59 ppm in the non-acetylated parent compound, mangiferin (5). Eight singlets between $\delta_H$ 1.80-2.53 ppm integrating each for three protons were assigned for $-\text{CH}_3$ protons of acetyl groups, which indicated the presence of the peracetylated derivative 6.

$^{13}$C NMR spectrum of compound 6 showed eight signals between $\delta_C$ 167.2-170.6 ppm, and eight signals between $\delta_C$ 20.3-21.4 ppm, which were assigned to eight C=O of the acetyl groups and to eight $-\text{CH}_3$ groups, respectively.

In $^{13}$C NMR spectrum of non-acetylated mangiferin (5), carbons C-1, 3, 6, and 7 were assigned to $\delta_C$ 161.8, 163.9, 154.1, and 143.8 ppm, respectively, whereas in compound 6 these carbons were shielded ($\delta_C$ 152.9, 154.4, 152.9, and 139.4 ppm). In contrast, carbons C-4 and C-5, in non-acetylated mangiferin (5), were respectively assigned to $\delta_C$ 93.3 and 102.7 ppm, whereas in compound 6 these carbons were deshielded ($\delta_C$ 111.7 and 112.7 ppm).

2.2.2. Mangiferin heptaacetate (7)

Structure elucidation of mangiferin heptaacetate (7, Figure 16) was established by IR and NMR ($^1$H and $^{13}$C) and is in accordance with those reported.99

![Figure 16 – Mangiferin heptaacetate (7).](image-url)

The presence of acetyl groups was suggested by the observation of two strong bands at 1779 and 1746 cm$^{-1}$ typical of C=O ester stretch. Additionally, a large band of stretching vibration of the O-H bond at 3443 cm$^{-1}$ suggested at least one free hydroxyl (Table 4).
Table 4 – IR data of compound 7.

<table>
<thead>
<tr>
<th>Groups</th>
<th>$\nu$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H</td>
<td>3443</td>
</tr>
<tr>
<td>C-H aliphatic</td>
<td>2919, 2850</td>
</tr>
<tr>
<td>C=O ester</td>
<td>1779, 1746</td>
</tr>
<tr>
<td>C=O ketone</td>
<td>1647</td>
</tr>
<tr>
<td>C=C aromatic</td>
<td>1619, 1467</td>
</tr>
<tr>
<td>C-O</td>
<td>1229</td>
</tr>
</tbody>
</table>

$\nu$ - wavenumber

$^1$H and $^{13}$C NMR data for compound 7 are also presented in APPENDIX I.

Characteristic signals of the aromatic protons H-4, H-5, and H-8 appeared as singlets at $\delta_H$ 6.76, 7.42, and 8.06 ppm, respectively, whereas the corresponding signals of these protons appeared at $\delta_H$ 6.38, 6.87, and 7.38 ppm in the non-acetylated parent compound, mangiferin (5, also in APPENDIX I). Seven signals characteristic of aliphatic protons (the sugar moiety) were observed between $\delta_H$ 3.81-5.68 ppm, whereas the corresponding signals of these protons appeared between $\delta_H$ 3.09-4.59 ppm in the non-acetylated parent compound, mangiferin (5). Seven singlets between $\delta_H$ 1.78-2.44 ppm integrating each for three protons were assigned for -CH$_3$ protons of acetyl groups, which indicated the presence of the peracetylated derivative 7.

$^{13}$C NMR spectrum showed seven signals between $\delta_C$ 167.1-170.5 ppm, and seven signals between $\delta_C$ 20.4-22.7 ppm, which were assigned to seven C=O of the acetyl groups and to seven -CH$_3$ groups, respectively.

In $^{13}$C NMR spectrum of non-acetylated mangiferin (5), carbons C-3, 6, and 7 were assigned to $\delta_C$ 163.9, 154.1, and 143.8 ppm, respectively, whereas these carbons were shielded ($\delta_C$ 153.9, 148.4, and 139.4 ppm) in compound 7. In contrast, carbons C-4 and C-5, in non-acetylated mangiferin (5), were respectively assigned to $\delta_C$ 93.3 and 102.7 ppm, whereas these carbons were deshielded ($\delta_C$ 110.6 and 112.9 ppm) in compound 7.
2.2.3. Naringin-di-deoxycholate (10)

Structure elucidation of naringin-di-deoxycholate (10, Figure 17) was established by IR, NMR (\(^1\)H and \(^{13}\)C), HSQC, and HMBC.

![Structure of Naringin-di-deoxycholate (10)](image)

**Figure 17** – Naringin-di-deoxycholate (10).

IR spectrum showed a band typical of C=O ester stretch at 1737 cm\(^{-1}\) (Table 5).

### Table 5 – IR data of compound 10.

<table>
<thead>
<tr>
<th>Groups</th>
<th>(\nu) cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H</td>
<td>3415</td>
</tr>
<tr>
<td>C-H aliphatic</td>
<td>2933, 2865</td>
</tr>
<tr>
<td>C=O ester</td>
<td>1737</td>
</tr>
<tr>
<td>C=O ketone</td>
<td>1642</td>
</tr>
<tr>
<td>C-O</td>
<td>1087</td>
</tr>
</tbody>
</table>

\(\nu\) - wavenumber

\(^1\)H and \(^{13}\)C NMR data for compound 10 are presented in **APPENDIX IV**.

\(^1\)H NMR spectrum showed signals of the flavanone glycoside position and of the steroid scaffold. The number of protons indicated the presence of two molecules of compound 9. Typical signals of the aromatic protons H-2', H-6' and H-3', H-5' appeared as duplet at \(\delta_H\) 7.56 and 7.17 ppm, respectively, whereas the corresponding signals of these protons appeared at \(\delta_H\) 7.33 and 6.80 ppm in naringin (8) indicating that molecular modification occurred at 4'-position. Additionally, \(^1\)H NMR spectrum showed a singlet at \(\delta_H\) 12.03 ppm that was assigned for 5-OH.
$^{13}$C NMR spectra showed two signals at $\delta_c$ 173.2 and 172.2 ppm that indicate the presence of two carbons typical of C=O ester groups. Carbon C-4' was assigned to $\delta_c$ 150.7 ppm, whereas the same carbon was assigned to $\delta_c$ 157.9 ppm in compound 8.

The assignments of carbon atoms directly bonded to proton atoms were achieved from HSQC experiments and the chemical shifts of the carbon atoms not directly bonded to proton atoms were deduced from HMBC correlations (Figure 18).

Figure 18 – Main connectivities found in HMBC for compound 10.

2.2.4. Methyl 4’-naringin acetate (11)

Structure elucidation of methyl 4’-naringin acetate (11, Figure 19) was established for the first time by IR, NMR ($^1$H and $^{13}$C), HSQC, and HMBC.

Figure 19 – Methyl 4’-naringin acetate (11).
IR spectrum of compound 11 showed a band at 1754 cm\(^{-1}\) characteristic of a C=O ester stretch which is in accordance with the molecular modification performed (Table 6).

<table>
<thead>
<tr>
<th>Groups</th>
<th>(\tilde{\nu} \ \text{cm}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H</td>
<td>3420</td>
</tr>
<tr>
<td>C-H aliphatic</td>
<td>2922</td>
</tr>
<tr>
<td>C=O ester</td>
<td>1754</td>
</tr>
<tr>
<td>C=O ketone</td>
<td>1632</td>
</tr>
<tr>
<td>C=C aromatic</td>
<td>1511</td>
</tr>
</tbody>
</table>

\(\tilde{\nu}\) - wavenumber

Table 6 – IR data of compound 11.

\(^1\)H and \(^{13}\)C NMR data of compound 11 is presented in APPENDIX II.

\(^1\)H and \(^{13}\)C NMR spectra of compound 11 showed signals that indicate the presence of only one carbomethoxymethyl group, namely signals corresponding to the methyl group (\(\delta_H 3.71\) and \(\delta_C 51.9\) ppm) and to the methylene bridge (\(\delta_H 4.83\) and \(\delta_C 65.4\) ppm).

Characteristic signals of the aromatic protons H-2', H-5', and H-3', H-6' appeared at \(\delta_H 7.46\) and 7.02-6.97 ppm, respectively, whereas the corresponding signals of the same protons appeared at \(\delta_H 7.33\) and 6.80 ppm in the parent compound, naringin (8, also in APPENDIX II). Signals of the hydroxyl protons of the sugar moiety (\(\delta_H 4.83\)-5.15 ppm) and the hydroxyl proton at C-5 (\(\delta_H 12.05\) ppm) were also observed in the \(^1\)H NMR spectrum of compound 11.

The assignments of carbon atoms directly bonded to proton atoms were achieved from HSQC experiments and the chemical shifts of the carbon atoms not directly bonded to proton atoms were deduced from HMBC correlations (Figure 20). The position of the methylacetate group on naringin was further confirmed by the correlations observed in HMBC spectrum between the signal of methylene protons (\(\delta_H 4.83\) ppm) and the signal of C-4' (\(\delta_C 157.9\) ppm) (Figure 20).
Figure 20 – Main connectivities found in HMBC for compound 11.
2.2.5. 4’-Naringin acetic acid (12)

Structure elucidation of 4’-naringin acetic acid (12, Figure 21) was established for the first time by IR and NMR (\(^1\)H and \(^{13}\)C).

![Structure of 4’-Naringin acetic acid (12).](image)

Figure 21 – 4’-Naringin acetic acid (12).

IR spectrum of compound 12 showed a band at 1735 cm\(^{-1}\) from the C=O carboxylic acid stretch suggesting the presence of the carboxylic acid group (Table 7).

<table>
<thead>
<tr>
<th>Groups</th>
<th>(\nu) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H</td>
<td>3438</td>
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<tr>
<td>C-H aliphatic</td>
<td>2919</td>
</tr>
<tr>
<td>C=O carboxylic acid</td>
<td>1735</td>
</tr>
<tr>
<td>C=O ketone</td>
<td>1628</td>
</tr>
<tr>
<td>C=C aromatic</td>
<td>1512</td>
</tr>
</tbody>
</table>

\(\nu\) - wavenumber

\(^1\)H and \(^{13}\)C NMR data for compound 12 are also presented in APPENDIX II.

\(^1\)H NMR spectrum showed a signal at \(\delta_H\) 13.89 ppm indicating the presence of an acidic proton in the carboxylic acid group. When comparing with methyl 4’-naringin acetate (11), the \(^1\)H NMR and \(^{13}\)C NMR spectra of compound 12 did not show a singlet signal around \(\delta_H\) 3.71 ppm neither a signal corresponding to a -CH\(_3\) carbon of an acetate group.
2.2.6. Succinimido deoxycholate (13)

Structure elucidation of DOCA-NHS (13, Figure 22) was established by IR and NMR (\(^1\)H and \(^{13}\)C). The \(^1\)H NMR is in accordance with those reported.\(^{40}\)

![Diagram of DOCA-NHS](image)

**Figure 22** – Succinimido deoxycholate (DOCA-NHS, 13).

IR spectrum of compound 13 showed bands at 1811, 1781, and 1741 cm\(^{-1}\) typical of the C=O stretch vibration (**Table 8**).

<table>
<thead>
<tr>
<th>Groups</th>
<th>(\nu) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H</td>
<td>3432</td>
</tr>
<tr>
<td>C-H aliphatic</td>
<td>2938, 2863</td>
</tr>
<tr>
<td>C=O (NHS)</td>
<td>1811, 1781</td>
</tr>
<tr>
<td>C=O ester</td>
<td>1741</td>
</tr>
</tbody>
</table>

\(\nu\) - wavenumber

\(^1\)H and \(^{13}\)C NMR data for compound 13 are presented in **APPENDIX III**.

\(^1\)H NMR spectrum showed a multiplet signal integrating for four protons between \(\delta_H\) 2.82-2.87 ppm that was assigned for the aliphatic protons of NHS.

\(^{13}\)C NMR spectrum showed a signal at \(\delta_C\) 169.2 ppm that was assigned to the two C=O of NHS. Additionally, when comparing with DOCA (9), \(^1\)H NMR spectra of compound 13 did not show a singlet signal of the acidic proton between \(\delta_H\) 12-13 ppm.
2.2.7. $N$-Deoxycholylethylenediamine (14)

Structure elucidation of DOCA-NH$_2$ (14, Figure 23) was established for the first time by IR and NMR ($^1$H and $^{13}$C). Although compound 14 is already described in the literature, this is the first complete characterization of compound 14.

![Diagram of $N$-Deoxycholylethylenediamine (DOCA-NH$_2$, 14).](image)

Figure 23 – $N$-Deoxycholylethylenediamine (DOCA-NH$_2$, 14).

The IR spectrum showed two bands at 1558 and 1628 cm$^{-1}$ of the N-H bend, and a band at 671 cm$^{-1}$ of the N-H wag (Table 9). Overlapping occurred in the observed position of N-H and O-H stretching frequencies so the presence of a primary amine is difficult to confirm.

<table>
<thead>
<tr>
<th>Groups</th>
<th>$\nu$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H/N-H stretch</td>
<td>3600-3200</td>
</tr>
<tr>
<td>C-H aliphatic</td>
<td>2926, 2861</td>
</tr>
<tr>
<td>C=O amide</td>
<td>1694</td>
</tr>
<tr>
<td>N-H bend</td>
<td>1628, 1558</td>
</tr>
<tr>
<td>N-H wag</td>
<td>671</td>
</tr>
</tbody>
</table>

$\nu$ - wavenumber
\(^1\)H and \(^{13}\)C NMR data for compound 14 are presented in APPENDIX III.

\(^1\)H NMR spectrum showed a triplet integrating for one proton at \(\delta_H 7.72\) ppm typical of a secondary amine proton and a quartet integrating for four protons at \(\delta_H 3.01\) ppm that was assigned to the methylene protons of the EDA aliphatic chain.

\(^{13}\)C NMR spectrum showed two signals from the carbons of the methylene groups of the EDA aliphatic chain (\(\delta_C 41.4\) and 41.6 ppm).

2.2.8. 4’-Naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15)

Structure elucidation of 4’-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15, Figure 24) was established for the first time by IR and NMR (\(^1\)H and \(^{13}\)C).

IR spectrum showed bands of the O-H stretch at 3443 cm\(^{-1}\) (Table 10). The band of the N-H amide, the C=O ketone stretch, and the C=O amide stretch were overlapped.

![Figure 24 – 4’-Naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15).](image)

<table>
<thead>
<tr>
<th>Groups</th>
<th>(\nu) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H</td>
<td>3443</td>
</tr>
<tr>
<td>C-H aliphatic</td>
<td>2924</td>
</tr>
<tr>
<td>C=O ketone/amide/N-H</td>
<td>around 1633</td>
</tr>
</tbody>
</table>

\(\nu\) - wavenumber

Table 10 – IR data of compound 15.
$^1$H and $^{13}$C NMR data for compound 15 are presented in **APPENDIX IV**.

$^1$H NMR spectrum showed singlet signals of two protons of the amide bonds between δ$_H$ 7.79-7.87 ppm and at δ$_H$ 8.17 ppm which indicate the success of the coupling reaction between compound 12 and compound 14.

$^{13}$C NMR spectrum showed two signals at at δ$_C$ 174.3 and 168.6 ppm corresponding to the C=O of amide bonds.

2.2.9. 4'-Naringin (N-(2-deoxycholan-24-amidoethyl))acetamide persulfate (16)

Structure elucidation of 4'-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide persulfate (16, **Figure 25**) was established for the first time by IR, NMR ($^1$H and $^{13}$C), HSQC, and HMBC.

![Figure 25](image-url) - 4'-Naringin (N-(2-deoxycholan-24-amidoethyl))acetamide persulfate (16).

IR spectrum showed bands from the sulfate groups at 1240 cm$^{-1}$ (S=O), 1058 cm$^{-1}$ (C-O-S), and 810 cm$^{-1}$ (S-O) (**Table 11**).
Table 11 – IR of compound 16.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ν (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-H aliphatic</td>
<td>2921, 2863</td>
</tr>
<tr>
<td>C=O ketone/amide</td>
<td>1632</td>
</tr>
<tr>
<td>N-H bend</td>
<td>1558</td>
</tr>
<tr>
<td>S=O</td>
<td>1240</td>
</tr>
<tr>
<td>C-O-S</td>
<td>1058</td>
</tr>
<tr>
<td>S-O</td>
<td>810</td>
</tr>
</tbody>
</table>

ν - wavenumber

¹H and ¹³C NMR data for compound 16 is presented in **APPENDIX IV**.

¹H NMR spectrum of compound 16 did not show signals in the typical region of hydroxyl proton signals (δH 4.53-5.36 ppm) when compared to compound 15. Twelve signals characteristic of aliphatic protons (the sugar moiety) were observed between δH 3.70-4.75 ppm in compound 16, whereas the corresponding signals of these protons appeared between δH 3.35-3.77 ppm in the non-sulfated parent compound, 4'-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15, also in **APPENDIX IV**). Signals of H-3 and H-12 of the steroid moiety appeared at δH 3.70-4.01 and 4.42-4.53 ppm, respectively, whereas the corresponding signals of these protons appeared at δH 3.70-4.01 and 4.42-4.53 ppm in the non-sulfated parent compound, 4'-naringin (N-(2-deoxycholan-24-amidoethyl))acetamide (15).

¹³C NMR spectrum showed signals of the C=O group of the amide bond at δC 173.1 and 167.8 ppm. Signals of the ethyl linker between the amide bonds were observed at δC 38.4 and 38.2 ppm, and of the 4'-OCH₂ at δC 67.0 ppm.

The assignments of the carbon atoms directly bonded to proton atoms were achieved from HSQC experiments and the chemical shifts of the carbon atoms not directly bonded to proton atoms were deduced from HMBC correlations (Figure 26).
Figure 26 - Main connectivities found in HMBC for compound 16.
2.2.10. **3,6-Bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (19)**

Structure elucidation of 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (19, Figure 27) was established for the first time by IR, NMR (\(^1\)H and \(^{13}\)C), HSQC and HMBC.

![Figure 27](image)

**Figure 27** – 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (19).

IR spectrum showed one band at 1745 cm\(^{-1}\) of C=O ester stretch (Table 12) and also a band at 2957 cm\(^{-1}\) of C-H stretch that suggest the presence of a sugar moiety.

**Table 12** – IR data of compound 19.

<table>
<thead>
<tr>
<th>Groups</th>
<th>(\nu) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-H aliphatic</td>
<td>2957</td>
</tr>
<tr>
<td>C=O ester</td>
<td>1745</td>
</tr>
<tr>
<td>C=O ketone/amide</td>
<td>1660</td>
</tr>
<tr>
<td>N-H bend</td>
<td>1609</td>
</tr>
</tbody>
</table>

\(\nu\) - wavenumber

\(^1\)H and \(^{13}\)C NMR data for compound 19 are presented in **APPENDIX V**.

The success of the molecular modification performed was confirmed by \(^1\)H NMR and \(^{13}\)C NMR that showed signals of the triazole ring (\(\delta\_C\) 125.4 ppm, \(\delta\_H\) 8.15 ppm, and \(\delta\_C\) 141.5 ppm of CH=C, respectively) and of the sugar moiety (\(\delta\_H\) 1.71-2.02 ppm and \(\delta\_C\) 20.2-22.6 ppm of the -CH\(_3\) groups, and \(\delta\_C\) 169.1-170.1 ppm of the C=O of the acetyl groups).
Assignments of the carbon atoms directly bonded to proton atoms were achieved from HSQC experiments and the chemical shifts of the carbon atoms not directly bonded to proton atoms were deduced from HMBC correlations (Figure 28).

Figure 28 – Main connectivities found in HMBC for compound 19.

2.2.11. 3,6-Bis(1-(2-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (20)

Structure elucidation of 3,6-bis(1-(2-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (20, Figure 29) was established for the first time by IR, NMR (1H and 13C), HSQC and HMBC.
Figure 29 - 3,6-Bis(1-(2-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (20).

IR spectrum of compound 20 did only present a band at 1648 cm⁻¹ typical of the C=O ketone stretch which suggest that acetyl groups were removed (Table 13).

<table>
<thead>
<tr>
<th>Groups</th>
<th>ν (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H</td>
<td>3415</td>
</tr>
<tr>
<td>C-H aliphatic</td>
<td>2919</td>
</tr>
<tr>
<td>C=O ketone/amide</td>
<td>1648</td>
</tr>
<tr>
<td>N-H bend</td>
<td>1610</td>
</tr>
</tbody>
</table>

ν - wavenumber

Table 13 – IR data of compound 20.

¹H and ¹³C NMR data for compound 20 are presented in APPENDIX V.

¹H NMR spectrum showed signals between δ_H 4.57-5.08 ppm which is in accordance with the presence of hydroxyl protons.

In ¹³C NMR spectrum of compound 19, carbons C-1', C-3', and C-4' were assigned to δ_C 99.8, 68.4, and 70.7 ppm, respectively, whereas these carbons were deshielded (δ_C 100.7, 70.5, and 74.1 ppm) in compound 20.

The assignments of the carbon atoms directly bonded to proton atoms were achieved from HSQC experiments and the chemical shifts of the carbon atoms not directly bonded to proton atoms were deduced from HMBC correlations (Figure 30).
2.2.12. 3,6-Bis(1-(2-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (21)

Structure elucidation of 3,6-bis(1-(2-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (21, Figure 31) was established for the first time by IR, NMR (¹H and ¹³C).
Figure 31 – 3,6-Bis(1-(2-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (21).

The IR spectrum showed bands of N-H bend and of N-H wag at 1609 and 841 cm⁻¹, respectively (Table 15). Overlapping occurred in the observed position of N-H and O-H stretching frequencies so the presence of a primary amine is difficult to confirm.

Table 14 – IR data of compound 21.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ν (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td></td>
</tr>
<tr>
<td>O-H</td>
<td>3362</td>
</tr>
<tr>
<td>C-H aliphatic</td>
<td>2920, 2880</td>
</tr>
<tr>
<td>C=O ketone/amide</td>
<td>1641</td>
</tr>
<tr>
<td>N-H bend</td>
<td>1609</td>
</tr>
<tr>
<td>N-H wag</td>
<td>841</td>
</tr>
</tbody>
</table>

ν - wavenumber

¹H and ¹³C NMR data for compound 21 are presented in APPENDIX V.

Comparing with the starting material, compound 20, the ¹H NMR spectrum of compound 21 did not show the signal in the typical region of the -CH₃ protons of the acetamide group (-NHCOCH₃). A singlet signal of two protons at δ_H 1.44 ppm was observed which indicate the presence of a primary amine.

2.2.13. 3,6-Bis(1-(2-(2-amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (22)
Structure elucidation of 3,6-bis(1-(2-(2-amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (22, Figure 32) was established for the first time by IR, NMR (\(^1\)H and \(^{13}\)C), HSQC, and HMBC.

*Figure 32 – 3,6-Bis(1-(2-(2-amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (22).*

IR spectrum showed bands at 1257 cm\(^{-1}\) (C-O-S), 1047 cm\(^{-1}\) (S=O), and 814 cm\(^{-1}\) (S-O) (Table 15) which suggests the presence of sulfate groups.

**Table 15 - IR data of compound 22.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>(\nu) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-H aliphatic</td>
<td>2917, 2847</td>
</tr>
<tr>
<td>C=O ketone/amide</td>
<td>1642</td>
</tr>
<tr>
<td>N-H bend</td>
<td>1619</td>
</tr>
<tr>
<td>C=O</td>
<td>1257</td>
</tr>
<tr>
<td>C-O-S</td>
<td>1047</td>
</tr>
<tr>
<td>S-O</td>
<td>814</td>
</tr>
</tbody>
</table>

\(\nu\) - wavenumber

\(^1\)H and \(^{13}\)C NMR of compound 22 are presented in APPENDIX V.

When comparing with the starting material, compound 21, signals of hydroxyl protons were not observed in the \(^1\)H NMR spectrum of compound 22. \(^1\)H NMR spectrum also showed a signal at \(\delta_{\text{H}}\) 1.73 ppm integrated for two protons which indicate that the amine group was not modified.
In $^{13}$C NMR spectrum of compound 21, carbons C-1', C-3', C-4', and C-6' were assigned to $\delta_{C}$ 101.5, 70.9, 76.5, and 61.1 ppm, respectively, whereas these carbons were shielded ($\delta_{C}$ 100.5, 70.5, 74.5, and 60.9 ppm), in compound 22.

The assignments of the carbon atoms directly bonded to proton atoms were achieved from HSQC experiments and the chemical shifts of the carbon atoms not directly bonded to proton atoms were deduced from HMBC correlations (Figure 33).

Figure 33 - Main connectivities found in HMBC for compound 22.
2.2.14. Diosmin peracetate (24)

Structure elucidation of diosmin peracetate (24, Figure 34) was established by IR and NMR (\(^{1}\)H and \(^{13}\)C). The \(^{1}\)H NMR is in accordance with those reported.\(^{129}\)

![Figure 34 – Diosmin peracetate (24).](image)

The IR spectrum showed a band at 1754 cm\(^{-1}\) (C=O ester stretch) that suggests the presence of acetyl groups (Table 16).

<table>
<thead>
<tr>
<th>Groups</th>
<th>(\nu) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-H aliphatic</td>
<td>2940</td>
</tr>
<tr>
<td>C=O ester</td>
<td>1754</td>
</tr>
<tr>
<td>C=O ketone</td>
<td>1644</td>
</tr>
<tr>
<td>C=C aromatic</td>
<td>1614, 1514, 1433</td>
</tr>
<tr>
<td>C-O</td>
<td>1289</td>
</tr>
<tr>
<td>C-H aromatic</td>
<td>982</td>
</tr>
</tbody>
</table>

\(\nu\) - wavenumber

\(^{1}\)H and \(^{13}\)C NMR data of compound 24 are presented in APPENDIX VI.

Characteristic signals of the aromatic protons H-2’, H-5’, and H-6’ appeared at \(\delta\)\(^{1}\)H 7.56, 7.08, and 7.72 ppm, respectively, whereas the corresponding signals of these protons appeared at \(\delta\)\(^{1}\)H 7.41, 7.10, and 7.54 ppm in the non-acetylated parent compound, diosmin (23, also in APPENDIX VI). Twelve signals characteristic of aliphatic protons (the sugar
moiety) were observed between $\delta_H$ 3.65-5.98 ppm, whereas the corresponding signals of these protons appeared between $\delta_H$ 3.13-5.03 ppm in the non-acetylated parent compound, diosmin (23). Eight singlets between $\delta_H$ 1.92-2.43 ppm integrating each for three protons were assigned for $-\text{CH}_3$ protons of acetyl groups, which revealed the formation of the peracetated derivative 23.

$^{13}C$ NMR spectrum showed eight signals between $\delta_C$ 168.8-170.2 ppm, and eight signals between $\delta_C$ 20.6-31.0 ppm, which were assigned to eight C=O of the acetyl groups and to eight $-\text{CH}_3$ groups, respectively.

In $^{13}C$ NMR spectrum of non-acetylated diosmin (23), carbons C-5 and 3' were assigned to $\delta_C$ 161.1 and 146.9 ppm, respectively, whereas these carbons were shielded ($\delta_C$ 159.8 and 140.1 ppm) in compound 24. In contrast, carbons C-3 and C-8, in non-acetylated diosmin (23), were respectively assigned to $\delta_C$ 105.7 and 95.1 ppm, whereas these carbons were deshielded ($\delta_C$ 109.0 and 97.8 ppm) in compound 24.

2.2.15. Rutin peracetate (26)

Structure elucidation of rutin peracetate (26, Figure 35) was established by IR, NMR ($^1H$ and $^{13}C$), HSQC and HMBC. The NMR ($^1H$ and $^{13}C$) is in accordance with those reported.$^{129,130}$

![Figure 35 – Rutin peracetate (26).](image)

IR spectrum showed two bands at 1754 and 1781 cm$^{-1}$ (C=O ester stretch) suggesting the presence of acetyl groups (Table 17).
Table 17 – IR data of compound 26.

<table>
<thead>
<tr>
<th>Groups</th>
<th>$\nu$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-H aliphatic</td>
<td>2940</td>
</tr>
<tr>
<td>C=O ester</td>
<td>1781, 1754</td>
</tr>
<tr>
<td>C=O ketone</td>
<td>1628</td>
</tr>
<tr>
<td>C=C aromatic</td>
<td>1505, 1477, 1435</td>
</tr>
<tr>
<td>C-O</td>
<td>1145</td>
</tr>
</tbody>
</table>

$\nu$ - wavenumber

$^1$H and $^{13}$C NMR data for compound 26 are presented in APPENDIX VII.

Characteristic signals of the aromatic protons H-2', H-5', and H-6' appeared at $\delta_H$ 7.91, 7.34, and 7.96 ppm, respectively, whereas the corresponding signals of these protons appeared at $\delta_H$ 7.54, 6.85, and 7.56 ppm in the non-acetylated parent compound, rutin (25, also in APPENDIX VII). Twelve signals characteristic of aliphatic protons (the sugar moiety) were observed between $\delta_H$ 3.26-5.43 ppm, whereas the corresponding signals of these protons appeared between $\delta_H$ 3.06-5.37 ppm in the non-acetylated parent compound, rutin (25). Ten singlets between $\delta_H$ 1.60-2.44 ppm integrating each for three protons were assigned for -CH$_3$ protons of acetyl groups, which revealed the formation of the peracetylated derivative 26.

$^{13}$C NMR spectrum showed eight signals between $\delta_C$ 167.8-170.2 ppm, and eight signals between $\delta_C$ 20.6-21.2 ppm, which were assigned to seven C=O of the acetyl groups and to seven -CH$_3$ groups, respectively.

In $^{13}$C NMR spectrum of non-acetylated rutin (25), carbons C-5, 7, 3', and 4' were assigned to $\delta_C$ 161.3, 164.1, 144.8, and 149.5 ppm, respectively, whereas these carbons were shielded ($\delta_C$ 150.2, 153.9, 141.8, and 144.1 ppm) in compound 26. In contrast, carbons C-6 and C-8, in non-acetylated rutin (25), were respectively assigned to $\delta_C$ 98.8 and 100.8 ppm, whereas these carbons were deshielded ($\delta_C$ 113.4 and 109.1 ppm) in compound 26.

The assignments of the carbon atoms directly bonded to proton atoms were achieved from HSQC experiments and the chemical shifts of the carbon atoms not directly bonded to proton atoms were deduced from HMBC correlations (Figure 36).
2.2.16. Naringin persulfate (27)

Structure elucidation of naringin persulfate (27, Figure 37) was established for the first time by IR, NMR (\(^1\)H and \(^{13}\)C), HSQC, HMBC, and high resolution mass spectrometry (HRMS).

IR spectrum showed bands at 1254 cm\(^{-1}\) (S=O), 1052 cm\(^{-1}\) (C-O-S), and 810 cm\(^{-1}\) (S-O) suggesting the presence of sulfate groups (Table 18).
Table 18 – IR data of compound 27.

<table>
<thead>
<tr>
<th>Groups</th>
<th>$\nu$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td></td>
</tr>
<tr>
<td>C=O ketone</td>
<td>1637</td>
</tr>
<tr>
<td>C=C aromatic</td>
<td>1518</td>
</tr>
<tr>
<td>S=O</td>
<td>1254</td>
</tr>
<tr>
<td>C-O-S</td>
<td>1052</td>
</tr>
<tr>
<td>S-O</td>
<td>810</td>
</tr>
</tbody>
</table>

$\nu$ - wavenumber

$^1$H and $^{13}$C NMR data for compound 27 are presented in APPENDIX II.

Characteristic signals of the aromatic protons H-2’, H-5’ and H-3’, H-6’ appeared as duplets at $\delta_H$ 7.58 and 7.17 ppm, respectively, whereas the corresponding signals of these protons appeared at $\delta_H$ 7.33, and 6.80 ppm in the non-sulfated parent compound, naringin (8, also in APPENDIX II). Twelve signals characteristic of aliphatic protons (the sugar moiety) were observed between $\delta_H$ 3.71-5.31 ppm, whereas the corresponding signals of these protons appeared between $\delta_H$ 3.15-5.14 ppm in the non-sulfated parent compound, naringin (8).

In $^{13}$C NMR spectrum of non-sulfated naringin (8), carbons C-5 and C-4’ were assigned to $\delta_C$ 162.9 and 157.9 ppm, respectively, whereas these carbons were shielded ($\delta_C$ 157.0 and 155.6 ppm) in compound 27. In contrast, carbons C-6 and C-8, in non-sulfated naringin (8), were respectively assigned to $\delta_C$ 96.3 and 95.2 ppm, whereas these carbons were deshielded ($\delta_C$ 97.1 and 96.0 ppm) in compound 27.

The assignments of the carbon atoms directly bonded to proton atoms were achieved from HSQC experiments and the chemical shifts of the carbon atoms not directly bonded to proton atoms were deduced from HMBC correlations (Figure 38).
Figure 38 - Main connectivities found in HMBC for compound 27.
2.3. BIOLOGICAL ACTIVITIES

2.3.1. Antitumor activity

Flavonoids and xanthones are privileged structures, what explains the wide range of biological activities that they display. Natural products such as xanthones and flavonoids are well known for their antitumor properties.\textsuperscript{82,131}

Acetylated derivatives possessing antitumor activity have been described in the literature.\textsuperscript{132-135}

Mangiferin heptaacetate (6), diosmin peracetate (26) and rutin peracetate (27) were evaluated \textit{in vitro} for growth-inhibitory effect on three human tumor cell lines: A375-C5 (malignant melanoma IL-1 insensitive), MCF-7 (breast adenocarcinoma), and NCI-H460 (bronchioalveolar carcinoma) using a sulforhodamine B assay (Table 19), a methodology that determines cell density, based on the measurement of cellular protein content. These studies were performed at CESPU by Patrícia Duarte and Patrícia Silva under supervision of Professor Hassan Bousbaa.

A pronounced growth-inhibitory effect was observed for compounds 6 and 26 against the three human tumor cell lines (Table 19). Compound 26 was more potent than compound 6 in all the three cell-lines.

Table 19 – Cell growth inhibition activity displayed by compounds 6, 24, and 26 on three human tumor cell lines.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>A375-C5</th>
<th>MCF-7</th>
<th>NCI-H460</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>56.12 ± 1.69</td>
<td>87.98 ± 2.73</td>
<td>95.79 ± 5.65</td>
</tr>
<tr>
<td>24</td>
<td>&gt;150</td>
<td>&gt;150</td>
<td>&gt;150</td>
</tr>
<tr>
<td>26</td>
<td>8.29 ± 2.98</td>
<td>18.98 ± 5.12</td>
<td>17.57 ± 2.46</td>
</tr>
</tbody>
</table>

\textsuperscript{Doxorubicin was used as positive control. GI\textsubscript{50} - concentration for 50\% of maximal inhibition of cell proliferation.}

Compounds 6 and 26, the most promising compounds, were further tested against three human tumor lines of glioblastoma. Compound 26 was also shown to have potent inhibitory effect on the human glioblastoma cell lines tested (Table 20).
Table 20 – Cell growth inhibition activity of compounds 6 and 26 on three human glioblastoma cell lines.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>GI50(μM)</th>
<th>U251</th>
<th>U373</th>
<th>U87MG</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>&gt;150</td>
<td>&gt;150</td>
<td>&gt;150</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>21.57 ± 3.03</td>
<td>18.77 ± 2.01</td>
<td>13.13 ± 0.68</td>
<td></td>
</tr>
</tbody>
</table>

Doxorrubicin was used as positive control. GI50 - concentration for 50% of maximal inhibition of cell proliferation.

Compound 26 was the only compound that showed potent inhibitory effects against all the human tumor cell lines tested. These results stimulate the future synthesis of other polyphenolic compounds to establish other structure-activity relationships. Although the number of derivatives investigated was very reduced, it is possible to infer that the position of the sugar moiety seems to be critical for the inhibitory activity.

2.3.2. Anticoagulant activity

4’-Naringin (N-(2-deoxycholan-24-amidoethyl))acetamide persulfate (16), 3,6-bis(1-(2-(2-amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (22), and naringin persulfate (27) were screened in vitro for anticoagulant activity by the three classical coagulation times: APTT, PT, and TT (Figure 39). These clotting assays are commonly used in clinical laboratory for monitoring anticoagulant drugs in patients and in research laboratories to test anticoagulant activity of new compounds.

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69
Figure 39 - Representation of the coagulation cascade and the classical clotting assays.

APTT is one of the most used clotting assays and measures the activity of a large number of factors of the intrinsic and common pathways of the coagulation (Figure 39). This assay is performed using plasma previously treated with sodium citrate to prevent coagulation. A negative charged activator is added to the citrated plasma and addition of calcium initiates the coagulation process. The APPT is measured after the activation of factor XII until the formation of a stable clot.

PT is widely used in health care to diagnose the risk of bleeding and to monitor oral anticoagulation therapy. The PT test specifically measures the activity of coagulation factors of the extrinsic and common pathway (Figure 39). The test involves re-calcification of plasma once the sample has been incubated with the tissue factor source (apoprotein and phospholipid). The PT is measured after the activation of FXa until the formation of a stable clot.

TT measures the time required for formation of a stable clot, following addition of thrombin to citrated plasma. The TT test is commonly used to diagnose deficiencies in fibrinogen (Figure 39) and measures the time since the addition of thrombin until the formation of a stable clot.

i) **BILE ACID CONJUGATE**

The effects of persulfated naringin-deoxycholic acid conjugate 16 and non-conjugated naringin persulfate (27) on APTT, PT, and TT are represented in Figure 40. The anticoagulant activity of the parent non-sulfated conjugate (15) and non-sulfated naringin 70
(8) was not possible to test due to their low water solubility. The % of dimethylsulfoxide (DMSO) necessary to solubilize the non-sulfated molecules resulted in precipitation of plasma proteins.

Compound 16 showed potent anticlotting activity (Table 21). In the presence of 1 mM of compound 16, the formation of the fibrin clot was completely inhibited i.e., was not observed even after 180 s (APTT), 120 s (PT), and 240 s (TT) (Figure 40). At 160 μM, the clot formation after the activation of factor XII (APTT) was still inhibited and the TT was prolonged above the double (Figure 40). The concentration required to double the APPT (APTT₂) was around 40 μM (Table 21). Additionally, compound 16 showed better anticoagulant activity when compared with other sulfated phenolic compounds previously synthesized in LQOF.⁷⁴, ⁷⁵, ¹³⁸

Non-conjugated naringin persulfate (27) also affected the APTT, PT, and TT in a dose-dependent manner (Figure 40), however higher concentrations were necessary to achieve the same ratio, comparing with compound 16. Complete inhibition of APTT was only observed at 1 mM and the concentration able to double the APTT was around 200 μM (Table 21). So, conjugate 16 is more potent anticoagulant than compound 27. The conjugation not only retained but also increased the anticoagulant activity of compound 27. This result was expected because hydroxyl groups of the steroid were also sulfated.

**Figure 40:**

A graph showing the anticoagulant activity of compounds 27 and 16, with APTT as the dependent variable. The x-axis represents the concentration (μM) of the compounds, ranging from 0.0256 to 1, and the y-axis represents the ratio of the compound to control, ranging from 0 to 14. The graph includes data points for each concentration level, indicating a significant difference (*) at each concentration tested. The graph also includes a legend indicating the compounds tested. The results show that compound 16 has a higher anticoagulant activity compared to compound 27 at all concentration levels tested.
Potential Orally-Active Heparin-Like Compounds: Synthesis and Anticoagulant Activity

Figure 40 - Dose-dependent effects of compounds 16 and 27 on APTT, PT, and TT clotting assays using human pooled plasma, expressed as ratio of clotting time in the presence/absence of compound. a clotting time values greater than 180s, b clotting time values greater than 120s, c clotting time values greater than 240s, * $P < 0.05$
Table 21 - Effects of sulfated compounds 16 and 27 on blood coagulation.

<table>
<thead>
<tr>
<th></th>
<th>16</th>
<th>27</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTT inhib.(^a) (x10(^{-3})M)</td>
<td>0.16</td>
<td>1</td>
</tr>
<tr>
<td>APTT (_2) (x10(^{-3})M)</td>
<td>0.0442 ± 0.0002</td>
<td>0.209 ± 0.005</td>
</tr>
<tr>
<td>PT inhib.(^b) (x10(^{-3})M)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>PT (_2) (x10(^{-3})M)</td>
<td>0.222 ± 0.050</td>
<td>-</td>
</tr>
<tr>
<td>TT inhib.(^c) (x10(^{-3})M)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>TT (_2) (x10(^{-3})M)</td>
<td>0.0864 ± 0.0007</td>
<td>-</td>
</tr>
</tbody>
</table>

The synthesis and anticoagulant activity of sulfated derivatives of deoxycholic acid (9) and 4'-naringin acetic acid (12) would be interesting to perform in order to compare with the conjugated compound 16.

ii) TRIAZOLE LINKED XANTHONOSIDE

The effects of 3,6-bis(1-(2-(2-amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (22) on APTT, PT, and TT are represented in Figure 41. The anticoagulant activity of non-sulfated compound 21 was not possible to test due to their low water solubility. The % of DMSO necessary to solubilize the non-sulfated compound resulted in precipitation of plasma proteins.

Prolongation of APTT, PT, and TT in the presence of compound 22 was observed in a dose-dependent manner. However, PT and TT were less affected than APTT. Sulfated derivative 22 exhibited an APTT\(_2\) of 129 ± 3 µM, PT\(_2\) of 739 ± 10 µM, and TT\(_2\) of 230 ± 3 µM. These values were higher than the previous obtained for the non-triazole 3,6-(O-β-glucopyranosyl) xanthone persulfate (2).75
Figure 41 - Dose-dependent effects of compound 22 on APTT, PT, and TT clotting assays using human pooled plasma, expressed as ratio of clotting time in the presence/absence of compound. * clotting time values greater than 180s. ** P < 0.05.
In contrast to bile acid conjugation strategy, the triazole introduction strategy seems to decrease the *in vitro* anticoagulant activity, although it is worthy to perform permeability studies with Ussing chamber to test if compound 22 is able to cross the epithelial membrane.
CHAPTER 3 - EXPERIMENTAL SECTION
3.1. GENERAL MATERIALS AND METHODS

Commercial available reagents were purchased from Sigma Aldrich Co. and from Fluka. 3,6-Bis(prop-2-yn-yl)oxy)-9H-xanthen-9-one was synthesized in Laboratório de Química Orgânica e Farmacêutica da Faculdade de Farmácia, Universidade do Porto. Solvents used were products pro analysis or HPLC grade of the firms Sigma-Aldrich and Fluka.

Reactions were controlled by TLC using Merck silica gel 60 (GF254) plates. Compounds were visually detected by absorbance at 254 and/or 365 nm and ferric chloride 10% in MeOH or ninhydrin 3 mg/ml in EtOH following heat activation. MW reactions were performed using a MicroSYNTH 1600 from Millestone (ThermoUnicam, Portugal) synthesizer in sealed and open reaction vessels.

Purification of compounds was carried out either by flash chromatography using Fluka silica gel 60 (0.04-0.063 mm) or preparative TLC using Merck silica gel 60 (GF254) plates. The qualitative purity of the synthesized compounds was determined by TLC using two different mobile phases.

Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were measured on an ATI Mattson Genesis series FTIR (software: WinFirst v.2.10) spectrophotometer in KBr microplates (cm⁻¹). ¹H and ¹³C NMR spectra were performed in University of Aveiro, Department of Chemistry and were taken in CDCl₃ and DMSO-d₆, at room temperature, on Bruker Avance 300 instrument (300.13 MHz for ¹H and 75.47 MHz for ¹³C). ¹³C NMR assignments were made by 2D HSQC and HMBC experiments (long-range C, H coupling constants were optimized to 7 and 1 Hz). Chemical shifts are expressed in ppm values relative to tetramethylsilane (TMS) as an internal reference. Coupling constants are reported in hertz (Hz). HRMS mass spectra were measured on a APEX III mass spectrometer, recorded as ESI (Electrospray) made in Centro de Apoio Científico e Tecnológico à Investigação (CACTI, University of Vigo, Spain).

Six human tumor cell lines were used to study antitumor activity: MCF-7 ER(+) (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and A375-C5 (melanoma), U251 (glioblastoma astrocytoma), U373 (glioblastoma astrocytoma) and U87MG glioblastoma astrocytoma).

APTT, PT, and TT tests were performed using an STA Evolution coagulometer. The following commercial reagents were used: 00308 (Stago, França) for the APTT, 00665 (Stago, França) for the PT and 00611 (Stago, França) for the TT.
3.2. SYNTHESIS

3.2.1. Synthesis of mangiferin peracetate (6)

Mangiferin (5, M3547, 0.2 g, 0.5 mmol) and iodine (0.009 g, 0.07 mmol) were mixed in acetic anhydride (7 mL) and the reaction was kept under MW irradiation (400W) at 130 °C for 15 minutes. After cooling, a saturated solution of sodium thiosulfate was added to transform iodine (dark yellow) into iodide (yellow). The crude product was extracted with CH$_2$Cl$_2$ and the organic layer was extracted with a saturated solution of NaHCO$_3$ twice, dried with anhydrous Na$_2$SO$_4$, and filtered. The solvent was evaporated under reduced pressure and the oil obtained was dissolved in ethyl acetate and poured into ice and a saturated solution of NaHCO$_3$ to afford 1,3,6,7-tetra-O-acetyl-2-C-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-9H-xanthen-9-one (6) as a yellow solid (0.294 g, 0.39 mmol, 78% yield). mp 143-147 °C (petroleum ether 60-80°C); IR (KBr) \( \nu_{\text{max}}: 3443, 2937, 1781, 1754, 1664, 1618, 1459, 1370, 1172 \text{cm}^{-1}; ^1\text{H} \text{NMR (CDCl}_3, 300.13 \text{MHz}) \delta: 8.00 (1\text{H, s, H-8}), 7.39 (1\text{H, s, H-5}), 7.25 (1\text{H, s, H-4}), 5.73 (1\text{H, t, J=9.7 Hz, H-2'}), 5.35-5.14 (2\text{H, m, H-3'} and H-4'), 4.86 (1\text{H, dd, J=4.9 and 10.1 Hz, H-1'}), 4.42 (1\text{H, dd, J=4.2 and 12.6 Hz, H-6}b), 4.05-3.98 (1\text{H, d, J=12.6 Hz, H-6}a), 3.84-3.80 (1\text{H, m, H-5}'), 2.53 (3\text{H, s, COCH}_3), 2.49 (3\text{H, s, COCH}_3), 2.34 (3\text{H, s, COCH}_3), 2.32 (3\text{H, s, COCH}_3), 2.07 (3\text{H, s, COCH}_3), 2.06 (3\text{H, s, COCH}_3), 2.03 (3\text{H, s, COCH}_3), 1.80 (3\text{H, s, COCH}_3); ^{13}\text{C} \text{NMR (CDCl}_3, 75.47 \text{MHz}) \delta: 174.0 (\text{C-9}), 170.6 (\text{OOCCH}_3), 170.5 (\text{OOCCH}_3), 170.3 (\text{OOCCH}_3), 169.6 (\text{OOCCH}_3), 169.2 (\text{OOCCH}_3), 168.5 (\text{OOCCH}_3), 168.0 (\text{OOCCH}_3), 167.7 (\text{OOCCH}_3), 167.2 (\text{OOCCH}_3), 157.4 (\text{C-4a}), 154.4 (\text{C-3}), 152.9 (\text{C-1 and C-6}), 147.7 (\text{C-10a}), 139.4 (\text{C-7}), 120.8 (\text{C-8}), 120.1 (\text{C-8a}), 118.3 (\text{C-2}), 112.7 (\text{C-5}), 112.1 (\text{9a}), 111.7 (\text{C-4}), 76.6 (\text{C-5}'), 74.4 (\text{C-3}'), 72.5 (\text{C-1}'), 69.4 (\text{C-2}'), 68.1 (\text{C-4}'), 61.9 (\text{C-6}'), 21.4 (\text{OOCCH}_3), 21.3 (\text{OOCCH}_3), 21.1 (\text{OOCCH}_3), 20.8 (\text{OOCCH}_3), 20.7 (\text{OOCCH}_3), 20.5 (\text{OOCCH}_3), 20.4 (\text{OOCCH}_3), 20.3 (\text{OOCCH}_3).

3.2.2. Synthesis of mangiferin heptaacetate (7)

Mangiferin (5, M3547, 0.2 g, 4.7 mmol) was mixed with acetic anhydride (10 mL) and the mixture was heated at 130 °C for 3.5 hours. After cooling, the solution obtained was poured into ice and a saturated solution of NaHCO$_3$, and extracted with CH$_2$Cl$_2$. Organic layer was dried with anhydrous Na$_2$SO$_4$, filtered and evaporated under reduced pressure. The crude oil obtained was further purified using flash chromatography (SiO$_2$; hexane:ethyl acetate, 7:3) to give 1-hydroxy-3,6,7-tri-O-acetyl-2-C-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-9H-xanthen-9-one (7) as a light yellow solid (0.0378 g, 0.053 mmol, 11% yield). mp 160-162 °C (hexane/ethyl acetate); IR (KBr) \( \nu_{\text{max}}: 3443, 2919, 2850, 1779, 78
3.2.3. Synthesis of naringin-di-deoxycholate (10)

DOCA (9, D2510, 0.338 g, 0.86 mmol, 1 eq.) and TBTU (Fluka 12806, 0.277 g, 0.86 mmol, 1 eq.) were dissolved in tetrahydrofuran (THF) and TEA (1.72 mmol, 0.24 mL, 2 eq.) was added. After 15 minutes, naringin (8, N1376, 0.5 g, 0.86 mmol) was added. The mixture was kept under a nitrogen atmosphere at room temperature for 72 hours. The solution obtained was poured into ice and the solid formed was filtered and purified by flash chromatography (SiO2; CHCl3:MeOH:HCOOH, 8:2:0.1) and then by preparative TLC (SiO2; CHCl3:MeOH:HCOOH, 8:2:0.1) to give the ester derivate 10 (0.025 g, 0.0188 mmol, 2.2%) as white solid. mp 182-185 °C (H2O); IR (KBr) vmax: 3415, 2933, 2865, 1737, 1642, 1448, 1374, 1087; 1H NMR (DMSO-d6, 300.13 MHz) δ: 12.03 (1H, s, 5-OH naringin), 7.56 (2H, d, J=8.1 Hz, H-2', H-6' naringin), 7.17 (2H, d, J=8.5 Hz, H-3', H-5' naringin), 6.15-6.09 (2H, m, H6, H-8 naringin), 5.70-5.61 (1H, m, H-2 naringin), 5.47 (1H, d, J=4.6 Hz, OH), 5.42 (2H, d, J=5.2 Hz, OH), 5.20-5.16 (1H, m, H-1'' neohesperidoside), 5.09 (1H, brd, H-1' neohesperidoside), 4.77-4.74 (1H, m, OH), 4.69 (1H, d, J=4.3 Hz, OH), 4.50-4.48 (3H, m, OH), 4.26 (2H, d, J=3.9 Hz, H-12), 4.18-4.16 (1H, m, OH), 4.07-4.00 (1H, m, OH), 3.82 (1H, d, J=2.9 Hz, 3-OH DOCA), 3.73-3.67 (6H, m, H-neohesperidoside, H-3 DOCA), 3.51-3.28 (2H, m, H-neohesperidoside, under water), 3.23-3.16 (4H, m, H-neohesperidoside), 2.89-2.87 (1H, m, H-3ax naringin), 2.83-2.81 (1H, m, H-3ax naringin), 2.28-2.18 (2H, H-23 DOCA), 1.82-1.24 (56H, m, H-steroidal DOCA), 1.17 (3H, d, J=6.1 Hz, 6''-CH3 neohesperidoside), 0.99 (6H, d, J=5.7, 21-CH3), 0.85 (6H, s, 19-CH3), 0.63 (3H, s, 18-CH3 DOCA), 0.56 (3H, s, 18-CH3 DOCA); 13C NMR (DMSO-d6, 75.47 MHz) δ: 197.0 (C-4 naringin), 173.2 (C-24 DOCA), 172.2 (C-24 DOCA), 163.0 (C-5 naringin), 162.8 (C-7 naringin), 150.7 (C-4' naringin), 136.2 (C-1' naringin), 128.0 (C-2', C-6' naringin), 122.0 (C-3', C-5' naringin), 100.7

Potential Orally Active Heparin-Like Compounds: Synthesis and Anticoagulant Activity
(C-1”), 97.0 (C-1” neohesperidoside, C-6/8 naringin), 95.0 (C-6/8 naringin) 77.0 (C-2 naringin), 71.1 (C-neohesperidoside, C-12 DOCA), 70.4 (C-neohesperidoside), 70.0 (C-neohesperidoside, C-3 DOCA), 68.0 (C-neohesperidoside), 47.5 (C-14 DOCA), 47.4 (C-14 DOCA), 46.2 (C-17 DOCA), 46.1, 46.0 (C-13 DOCA), 41.6 (C-5 DOCA, C-3 naringin), 40.3, 40.1, 39.8, 39.5, 39.2 (C-DOCA), 36.3 (C-8 DOCA), 35.7 (C-4 DOCA), 35.2 (C-20 DOCA), 35.0 (C-1), 33.9 (C-10 DOCA), 33.0, 30.8 (C-23 DOCA), 30.6 (C-23 DOCA), 30.3 (C-2 DOCA), 28.6 (C-11 DOCA), 27.3 (C-6 DOCA), 27.0 (C-16 DOCA), 26.1 (C-7 DOCA), 23.5 (C-15 DOCA), 23.1 (C-19 DOCA), 18.1 (C-6”’ neohesperidoside), 17.0 (C-21 DOCA), 16.7 (C-21 DOCA), 12.5 (C-18 DOCA), 12.4 (C-18 DOCA).

3.2.4. Synthesis of methyl 4’-naringin acetate (11)

Naringin (8, N1376, 1 g, 1.72 mmol) was dissolved in DMF (20 mL) and K$_2$CO$_3$ (0.285 g, 2.06 mmol, 1.2 eq.) was added. After 10 minutes, methyl bromoacetate (253 μL, 2.75 mmol, 1.6 eq.) was added. The mixture was kept at room temperature for 32 hours. The solution obtained was evaporated under reduced pressure and the solid was purified by flash chromatography (SiO$_2$:CHCl$_3$:acetone to give methyl 4’-(7-[[2-O-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-oxo) aceta (11) as an orange solid (0.206 g; 0.32 mmol; 18% yield). mp 166-170 °C (CHCl$_3$:acetone); IR (KBr) νmax: 3420, 2922, 1754, 1632, 1511, 1439, 1216, 1176, 1078, 832, 668; 1H NMR (DMSO-d$_6$, 300.13 MHz) δ: 12.05 (1H, s, 5-OH), 7.46 (2H, dd, J=2.4 and 8.9 Hz, H-2’ and H6’), 7.02-6.97 (2H, m, H3’ and H-5’), 6.20 (1H, d, J=2.5 Hz, H-8), 6.13-6.08 (1H, m, H-6), 5.60-5.52 (1H, m, H-2), 5.15-5.10 (3H, m, H-1”, H-1”’ and OH), 4.91-4.87 (4H, m, OHs), 4.83 (2H, s, O-CH$_3$), 3.74-3.64 (3H, m, H-5”’, H-2”’”, and H-6”’ a), 3.71 (3H, s, O-CH$_3$), under water (4H, m, H-2””, H-3”’, H5” and H-6”’b), 3.23-3.15 (3H, m, H-3”’, H-4”’ and H-4”), 2.82-2.73 (2H, m, H-3), 1.16 (3H, d, J=6.2 Hz, 6”’-CH$_3$); $^{13}$C NMR (DMSO-d$_6$, 75.47 MHz) δ: 197.2 (C-4), 169.2 (COOCH$_3$), 164.8 (C-7), 163.0 (C-5), 162.8 (C-9), 157.9 (C-4’), 128.5 (C-1’), 128.4 (C-2’), 128.2 (C-6’), 115.1 (C-3’ and C-5’), 103.4 (C-10), 100.5 (C-1”), 97.3 (C-1””), 96.4 (C-6), 95.2 (C-8), 79.2 (C-2), 77.1 (C-2”’), 76.9 (C-5”), 76.1 (C-3”), 71.8 (C-4””), 70.5 (C-2””), 70.4 (C-3””), 69.6 (C-4””), 68.3 (C-5””), 65.4 (4’-OCH$_2$), 60.5 (C-6””), 51.9 (OCH$_3$), 45.4 (C-3), 18.1 (C-6””).

3.2.5. Synthesis of 4’-naringin acetic acid (12)

Compound 11 (0.207 g, 0.316 mmol) was dissolved in MeOH (4 mL) and sodium methoxide (0.5 M solution in MeOH, 3 mL, 1.5 mmol) was added. The mixture was kept at room temperature for 5 hours. After completion of the reaction, the solvent was evaporated
under reduced pressure and the product neutralized using a strong cationic exchange solid phase extraction column. H$_2$O was evaporated under reduced pressure to give (7-[[2-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-oxy) acetic acid (12) as a dark yellow solid (0.199 g; 0.313 mmol; 99% yield). mp 215 ºC dec (H$_2$O); IR (KBr) νmax: 3438, 2919, 1735, 1628, 1512, 1424, 1348, 1215, 1177, 1128, 1072, 813; ¹H NMR (DMSO-$d_6$, 300.13 MHz) δ: 13.89 (1H, s, COOH), 12.05 (1H, s, 5-OH), 7.44 (2H, dd, J=2.4 and 8.8 Hz, H-2' and H-6'), 6.93 (2H, d, J=8.8 Hz, H-3' and H-5'), 6.16-6.08 (2H, m, H-6 and H-8), 5.61-5.53 (1H, m, H-2), 5.17-5.05 (4H, m, H-1", H-1'", and 2OH), 4.62 (1H, d, J=3.5 Hz, OH), 4.57 (1H, d, J=4.4 Hz, OH), 4.51 (1H, m, OH), 4.43-4.38 (1H, m, OH), 3.74-3.64 (2H, m, H-6"a, H-5"'), 3.49-3.16 (1H, H-6"b), 3.25-3.16 (3H, m, H-2'", H-3'"), and H-4""), 2.82-2.71 (2H, m, H-3), 1.16 (3H, d, J=6.2 Hz, 6"-CH$_3$); ¹³C NMR (DMSO-$d_6$, 75.47 MHz) δ: 197.2 (C-4), 170.1 (C=O), 164.8 (C-7), 163.3 (C-5), 162.8 (C-9), 157.9 (C-4'), 128.5 (C-1'), 128.4 (C-2'), 128.3 (C-6'), 115.0 (C-3' and C-5'), 100.5 (C-1''), 103.3 (C-10), 97.4 (C-1"'), 96.3 (C-6), 95.2 (C-8), 78.9 (C-2), 77.1 (C-2''), 76.9 (C-5''), 71.8 (C-4''), 70.5 (C-2"'), 70.4 (C-3"'), 69.6 (C-4''), 68.3 (C-5''''), 65.3 (4'-OCH$_2$), 60.5 (C-6''), 42.0 (C-3), 18.1 (6""-CH$_3$).

3.2.6.  Synthesis of succinimido deoxycholate (13)

DOCA (9, D2510, 1 g, 2.12 mmol) was dissolved in THF (50 mL) and DCC (0.745 g, 3.2 mmol, 1.7 eq./COOH) and NHS (0.416 g, 3.2 mmol, 1.7 eq./COOH) were added. The mixture was kept under nitrogen atmosphere, at room temperature, overnight. After completion of the reaction, insolubilized dicyclohexylurea was removed by filtration. The filtrate was concentrated under reduced pressure, hexane was added, and 2,5-dioxopyrrolidin-1-yl (R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoate (13) insolubilized as a white solid (1.1 g, 2.25 mmol, 88% yield). IR (KBr) νmax: 3432, 2938, 2863, 1811, 1781, 1741, 1448, 1207, 1066, 1043, 994, 648 cm⁻¹; ¹H NMR (CDCl$_3$, 300.13 MHz) δ: 4.00 (1H, t, J= 2.8, 3-OH), 3.77-3.73 (1H, m, H-12), 3.98-3.56 (1H, m, H-3), 2.87-2.82 (4H, m, CH$_2$-CH$_2$, NHS), 2.67-2.55 (2H, m, H-23a and H-23b), 1.94-1.05 (32H, m, H-steroidal), 1.01 (3H, d, J=6.1 Hz, 21-CH$_3$), 0.91 (3H, s, 19-CH$_3$), 0.70 (3H, s, 18-CH$_3$); ¹³C NMR (CDCl$_3$, 75.47 MHz) δ: 169.1 (C-24), 169.2 (C=O, NHS), 73.1 (C-12), 71.8 (C-3), 48.2 (C-14), 47.1 (C-17), 46.6 (C-13), 42.1 (C-5), 36.4 (C-8), 36.0 (C-4), 35.2 (C-20), 34.9 (C-1), 34.1 (C-10), 33.7 (C-9), 30.5 (C-2, C-22, C-23), 28.0 (C-11), 27.4 (C-6), 27.1 (C-16), 26.1 (C-7), 25.6 (CH$_2$-CH$_2$, NHS), 23.6 (C-15), 23.2 (C-19), 17.3 (C-21), 12.7 (C-18).
3.2.7. Synthesis of N-deoxycholyethylene diamine (14)

Succinimido deoxycholate (13, 0.5 g, 1.02 mmol) was dissolved in DMF (2.5 mL) and EDA (5 mL) was added. The mixture was kept at room temperature for 24 hours. The solution obtained was poured into ice and [(3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-dihydroxy-13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (14) insolubilized as a white solid (0.361 g, 0.831 mmol, 81% yield). mp 120-123 °C (H2O); IR (KBr) vmax: 3628, 2926, 2861, 1694, 1628, 1558, 1506, 1447, 1374, 671; 1H NMR (DMSO-d6, 300.13 MHz) δ: 7.72 (1H, t, J=10.9, NH), 4.49 (1H, s, 3-OH), 4.21 (1H, brd, H-12), 3.79 (1H, s, H-3), 3.01 (4H, q, J=6.3 and 12.2, CH2-CH2), 2.13-2.04 (1H, m, H-23a), 2.00-1.90 (2H, m, H-23), 1.82-0.97 (31H, m, H-steroidal, -NH2), 0.92 (3H, d, J=6.2 Hz, 21-CH3), 0.84 (3H, s, 19-CH3), 0.59 (3H, s, 18-CH3); 13C NMR (DMSO-d6, 75.47 MHz) δ: 172.7 (C-17), 71.0 (C-12), 70.0 (C-3), 47.5 (C-14), 46.2 (C-17), 46.0 (C-13), 42.3 (C-5), 36.3 (C-8), 35.7 (C-4), 35.1 (C-1), C-20), 33.8 (C-10), 32.9 (C-9, C-22), 32.5 (C-23), 28.7 (C-2), 28.6 (C-11), 27.2 (C-6), 27.0 (C-16), 26.1 (C-7), 23.5 (C-15), 23.1 (C-19), 17.1 (C-21), 12.5 (C-18).

3.2.8. Synthesis of 4’-naringin (N-(2-deoxycholan-24-amidoethyl)acetamide (15)

Compound 12 (0.123 g, 0.192 mmol) and TBTU (Fluka 12806, 0.059 g, 0.385 mmol, 2 eq.) were dissolved in DMF (7 mL) and TEA (54 μL, 0.385 mmol; 2 eq.) was added. After 15 minutes, compound 14 (0.084 g, 0.192 mmol, 1 eq.) was added. The mixture was kept at room temperature for 48 hours. After the completion of the reaction the mixture was poured into ice and the solid was filtrated and washed with H2O, aqueous solution of HCl 1M, and then with H2O to give (4R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-dihydroxy-13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)-N-(2-(4-(7-[[2-O-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(4-hydroxyphenyl)-4'H-1-benzopyran-4-oxyl)acetamido)ethyl)pentanamide (15) as a yellow solid (0.149 g, 0.141 mmol, 73% yield). mp 214-219 °C (H2O); IR (KBr) vmax: 3443, 2924, 1633, 1384, 1176, 1076; 1H NMR (DMSO-d6, 300.13 MHz) δ: 12.06 (1H, s, 5-OH, naringin), 8.17 (1H, s, NH-amide), 7.87-7.79 (2H, m, H-2'/6', NH amide), 7.49-7.45 (1H, brd, H-2'/6' naringin), 7.03-6.99 (2H, brd, H-3', H-5'), 6.21-6.10 (2H, m, H-6, H-8 naringin), 5.50-5.54 (1H, m, H-2 naringin), 5.36-5.32 (1H, m, OH neohesperidoside), 5.18-5.10 (2H, m, H-1", H-1" neohesperidoside), 4.77-4.53 (6H, m, OH neohesperidoside, 3-OH DOCA, -OCH2-), 4.19 (1H, s, H-12 doca), 3.77-3.69 (4H, m, H-neohesperidoside, H-3 DOCA), 3.55-3.35 (6H, m, H-neohesperidoside, under water signal), 3.18-3.15 (4H, m, CH2CH2 EDA), 2.89-82
2.71 (2H, m, H-3 naringin), 2.07-1.91 (2H, m, H-23a, H23b DOCA), 1.75-1.14 (28H, m, H-steroidal, 6''''-CH₃ neohesperidoside), 0.91 (3H, d, J=5.9 Hz, 21-CH₃ DOCA) 0.84 (3H, s, 19-CH₃ DOCA), 0.59 (3H, s, 18-CH₃ DOCA); ¹³C NMR (DMSO-d₆, 75.47 MHz) δ: 174.3 (C=O amide), 168.6 (C=O amide), 164.8 (C-7), 163.0 (C-5), 128.9 (C-2', C-6' naringin), 115.6 (C-3', C-5', naringin), 100.9 (C-1''', naeohesperidoside), 97.4 (C-1''', neohesperidoside), 96.8 (C-6,8 naringin), 77.4 (C-2 naringin), 76.6 (C-neohesperidoside), 72.3 (C-neohesperidoside), 71.6 (C-neohesperidoside, C-12 DOCA), 70.7 (C-neohesperidoside), 70.5 (C-neohesperidoside, C-3 DOCA), 68.8 (C-neohesperidoside), 67.2 (-OCH₂-), 60.9 (C-6'', neohesperidoside), 47.8 (C-14, DOCA), 46.6 (C-17, DOCA), 46.4 (C-13, C-3 DOCA), 42.0 (C-5, DOCA), 38.5 (CH₂CH₂-EDA), 36.5 (C- DOCA), 36.0 (C-DOCA), 35.4 (C-DOCA), 34.2 (C-DOCA), 33.3 (C-DOCA), 32.9 (C-DOCA), 32.0 (C-DOCA), 31.9 (C-DOCA), 31.6 (C-23 DOCA), 30.4, 28.9 (C-DOCA), 27.6 (C-DOCA), 27.3 (C-DOCA), 26.5 (C-DOCA), 23.9 (C-15, DOCA), 23.5 (C-19, DOCA), 18.4 (6''''-CH₃, naringin, 17.4 (C-21, DOCA), 12.8 (C-18, DOCA).

3.2.9. Synthesis of 4''-naringin (N-(2-deoxycholan-24-amidoethyl)acetamide persulfate (16)

Compound 15 (0.138 g, 0.131 mmol) was dissolved in DMA (15 mL) and triethylamine-sulfur trioxide (SO₃:TEA) adduct (2.13 g, 11.8 mmol, 10 eq./OH) was added. The mixture was kept under MW irradiation (200W) at 100 °C for 2 hours. After cooling, TEA was added until the pH was 8 and the obtained solution was poured into acetone and left at 4°C for 24 hours. The crude oil formed was washed with acetone and ether to remove unreacted adduct and then was dissolved in aqueous solution of 30% NaOAc (2 mL). EtOH was added and the sodium salt of (4R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-di-O-sulfate-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)-N-(2-(2-(4-(2-[(2-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl)oxy]-2,3-dihydro-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-oxy)acetamido)ethyl)pentanamide (16) insolubilized as a light brown solid that was further purified by dialysis using a Spectra/Por 6 regenerated cellulose MWCO 1000 membrane (0.130 g, 0.066 mmol, 50% yield). m.p. 215-219 °C (EtOH); IR (KBr) vmax: 2921, 2863, 1632, 1558, 1240, 1058, 810; ¹H NMR (DMSO-d₆, 300.13 MHz) δ: 8.24 (1H, s, NH-amide), 7.90 (2H, brd, H-2’/6’ naringin, NH amide), 7.49 (1H, brd, H-2’/6’ naringin), 7.03 (2H, brd, H3’, H-5’ naringin), 6.19-5.98 (2H, m, H-6, H-8 naringin), 5.35-5.20 (3H, m, H-2 naringin, H-1'', H-1'' neohesperidoside), 4.75-4.68 (2H, m, H-neohesperidoside), 4.53-4.42 (6H, m, H-12 DOCA, H-neohesperidoside, -OCH₂-), 4.01-3.70 (6H, m, H-neohesperidoside, H-3 DOCA), 3.16-3.15 (4H, m, CH₂CH₂-EDA, H-
3.2.10. Synthesis of 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (19)

3,6-Bis(prop-2-yn-yloxy)-9H-xanthen-9-one (17, 0.183 g, 0.6 mmol) and 2-azidoethyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (18, A1750, 0.499 g, 1.20 mmol) were dissolved in THF/H2O (2:1, 20 mL) and sodium ascorbate (0.475 g, 2.40 mmol) and Cu2SO4·5H2O (0.3 g, 1.20 mmol) were added. The mixture was kept under MW irradiation (500 W) for 30 min at 70°C. After cooling, the reaction mixture was filtered, the THF of the filtrate was evaporated under reduced pressure, and the water suspension was extracted twice with ethyl acetate. A green solid was formed in the aqueous phase and after filtration 3,6-bis(1-(2-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)-9H-xanthen-9-one (19) was separated as an orange solid (0.497 g, 0.437 mmol, 73% yield). mp 239-241°C (EtOAc); IR (KBr) νmax: 2957, 1745, 1660, 1609, 1441, 1373; 1H NMR (DMSO-d6, 300.13 MHz) δ: 8.15 (1H, s, H-triazole), 8.09 (1H, d, J=8.9 Hz, H-1/8), 7.92 (1H, d, J=9.1 Hz, NH-amide), 7.34 (1H, d, J=2.3 Hz, H-4/5), 7.10 (1H, dd, J=2.3 and 9.0 Hz, H-2/7), 5.33 (2H, s, O-CH2-triazole), 5.07 (1H, t, J=9.8 Hz, H-5/5’), 4.85 (1H, t, J=9.9 Hz, H-3’/3’’), 4.70 (1H, d, J=8.5 Hz, H-1’/1’’), 4.60-4.57 (2H, m, N-CH2CH2-O), 4.21-4.02 (5H, m, H-4’/4’’), 6’/6’’a, H-6’/6’’b, N-CH2CH2-O), 3.76 (1H, q, J=9.7, H-2’/2’’), 2.02 (3H, s, COCH3), 1.97 (3H, s, COCH3), 1.91 (3H, s, COCH3), 1.71 (3H, s, NHOCH3); 13C NMR (DMSO-d6, 75.47 MHz) δ: 174.1 (C-9), 170.1 (COCH3), 169.7 (COCH3), 169.4 (COCH3), 169.1 (NHOCH3), 163.3 (C-3/6), 157.4 (C-2a/10a), 141.5 (CH=C triazole), 127.5 (C-1/8), 125.2 (CH=C triazole), 115.0 (C-8a/9a), 113.8 (C-2/7), 101.5 (C-4/5), 99.8 (C-1’/1’’), 72.5 (C-5’/5’’), 70.7 (C-4’/4’’), 68.4 (C-3’/3’’), 67.2 (N-CH2CH2-N), 50.7, 48.2 (C-3, 6), 45.7 (C-3, 6), 45.6 (C-3, 6), 41.9 (C-3, 6), 38.4 (CH2CH2-EDA), 38.2 (CH2CH2-EDA), 35.5 (C-4 DOCA), 35.3 (C-1 DOCA), 33.9 (C-10 DOCA), 33.5-31.6 (C-DOCA), 27.3 (C-6 DOCA), 27.1 (C-16 DOCA), 26.1 (C-7, DOCA), 24.5, 23.6 (C-15, DOCA), 23.2 (C-19, DOCA), 18.0 (C-6’’’), neohesperidoside), 17.4 (C-21, DOCA), 12.3 (C-18, DOCA).
61.9 (OCH₂-triazole), 61.7 (C-6'/6''), 52.8 (C-2'/2''), 49.4 (N-CH₂CH₂-O), 22.6 (NHCOCH₃), 20.5 (COCH₃), 20.4 (COCH₃), 20.3 (COCH₃).

3.2.11. Synthesis of 3,6-bis(1-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (20)

Compound 19 (0.244 g; 0.215 mmol) was suspended in MeOH (8 mL) and MeONa (1 mL) was added. The mixture was kept under stirring at room temperature for 3 hours. The mixture was filtered and 3,6-bis(1-(2-acetamido-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-y]methoxy)-9H-xanthen-9-one (20) was obtained as a light green solid (0.162 g; 0.183 mmol, 86% yield). m.p. 238-240 °C (MeOH); IR (KBr) vmax: 3415, 2919, 1648, 1610, 1444, 1375; ¹H NMR (DMSO-d₆, 300.13 MHz) δ: 8.18 (1H, s, H-triazole), 8.10 (1H, d, J=9.0 Hz, H-1'), 7.70 (1H, d, J=8.9 Hz, NH-amide), 7.35 (1H, d, J=2.1 Hz, H-4'/5'), 7.12 (1H, dd, J=2.1 and 12 Hz H-2/7), 5.33 (2H, s, OCH₂-triazole), 5.05 (1H, brd, OH); 5.01 (1H, brd, OH), 4.60-4.57 (3H, m, N-CH₂CH₂-O, OH), 3.32 (1H, d, J=8.4 Hz, H-1'/1''), 4.12-4.08 (1H, m, N-CH₂CH₂-O), 3.87-3.81 (1H, m, N-CH₂CH₂-O), 3.73-3.68 (1H, m, H-6'/6''). 3.92-3.40 (1H, m, H-2'/2''), 3.29-3.25 (1H, m, H-4'/4''), 3.18-3.04 (2H, m, H(3'/3' and H-5'/5'')) 1.76 (3H, s, NHCOCH₃); ¹³C NMR (DMSO-d₆, 75.47 MHz) δ: 174.1 (C-9), 169.3 (NHCOOCH₃), 163.3 (C-3/6), 157.4 (C4a/10a), 141.6 (CH=CH-triazole), 127.4 (C-1/C-8), 125.5 (CH=C-triazole), 115.2 (C-8a/9a), 113.8 (C-2/7), 108.4 (C-4/5), 100.7 (C-1'/1''), 77.29 (C-5'/5''), 74.1 (C-4'/4''), 70.5 (C-3'/3''), 66.5 (N-CH₂CH₂-N), 61.9 (O-CH₂-triazole), 61.0 (C-6'/6''), 55.1 (C-2'/C-2''), 49.6 (N-CH₂CH₂-O), 23.0 (NHCOOCH₃).

3.2.12. Synthesis of 3,6-bis(1-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-y]methoxy)xanthone (21)

Compound 20 (0.155 g, 0.18 mmol) was suspended in an aqueous solution of NaOH 20% (15 mL). The mixture was heated at 100°C for 4 hours. After cooling, the mixture was purified by dialysis using a Spectra/Por 6 regenerated cellulose MWCO 1000 membrane to give 3,6-bis(1-(2-amino-3,4,6-hydroxy-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-y]methoxy)-9H-xanthen-9-one (21) as a dark green solid (0.051 g, 0.064 mmol, 36% yield). mp 157-159 °C (H₂O); IR (KBr) vmax: 3362, 2920, 2880, 1641, 1609, 841; ¹H NMR (DMSO-d₆, 300.13 MHz) δ: 8.37 (1H, s, H-triazole), 8.08 (1H, d, J=8.9 Hz, H-1/8), 7.33 (1H, brd, H-4/5), 7.11 (1H, d, J=8.8 Hz, H-2/7), 5.35 (2H, s, OCH₂-triazole), 5.04 (1H, brd, OH), 4.96 (1H, s, OH), 4.62-4.55 (3H, m, N-CH₂CH₂-O, OH), 4.12-4.10 (2H, m, N-CH₂CH₂-N, H1'/1''), 3.91-3.87 (1H, m, N-CH₂CH₂), 3.71-3.67 (2H, m, H-6'/6'a,b), 3.11-3.04 (4H, m, H-2'/2'',H-3'/3'',H-4'/4'',H-5'/5'') 1.44 (2H, s, NH₂); ¹³C NMR (DMSO-d₆, 75.47
Potential Orally-Active Heparin-Like Compounds: Synthesis and Anticoagulant Activity

3.2.13. Synthesis of 3,6-bis(1-(2-(amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)xanthone (22)

Compound 21 (0.045 g, 0.05 mmol) was dissolved in DMA (5 mL) and the mixture was kept under MW irradiation for 15 minutes. Then, SO₂·TEA adduct (0.046 g, 0.057 mmol, 10 eq./OH and NH₃) was added. The reaction solution was kept MW irradiation (200W) at 100 ºC for 2 hours. After cooling, TEA was added until the pH was 8 and the obtained solution was poured into acetone and left at 4 ºC for 24 hours. The crude oil formed was washed with acetone and ether to remove unreacted adduct and then was dissolved in aqueous solution of 30% NaOAc (2 mL). EtOH was added and the sodium salt of 3,6-bis(1-(2-(amino-3,4,6-tri-O-sulfate-2-deoxy-β-D-glucopyranosyl)ethyl)-1H-1,2,3-triazole-4-yl)methoxy)-9H-xanthen-9-one (22) insolubilized as a dark brown solid that was further purified by dialysis using a Spectra/Por 6 regenerated cellulose MWCO 1000 membrane (0.055 g, 0.04 mmol, 78% yield); mp 192 ºC dec (H₂O); IR (KBr) vmax: 2917, 2847, 1642, 1619, 1257, 1047, 814; ¹H NMR (DMSO-d₆, 300.13 MHz) δ: 8.27 (1H, s, CH=C-triazole), 8.07 (1H, d, J=8.8 Hz, H-1/8), 7.35 (1H, brd, H4/5), 7.13 (1H, d, J=7.5 Hz, H-2/7), 5.33 (2H, s, OCH₂-triazole), 4.63-4.60 (2H, m, N-CH₂CH₂-O), 4.15-4.08 (3H, m, H-1′/1′′, N-CH₂CH₂-O), 3.84-3.30 (3H, m, H-2′/2′′, H-3′/3′′, H-4′/4′′ and H5′/5′′, under water), 1.73 (2H, s, NH₂); ¹³C NMR (DMSO-d₆, 75.47 MHz) δ: 174.2 (C-9), 163.4 (C3/6), 157.5 (C-4a/10a), 141.7 (CH=C-triazole), 127.5 (C-1/8), 125.5 (CH=C-triazole), 115.2 (C-8a/9a), 113.9 (C-2/7), 101.5 (C-4/5), 100.5 (C-1′/1′′), 76.9 (C-5′/5′′), 74.5 (C-4′/4′′), 70.5 (C-3′/3′′), 66.1 (N-CH₂CH₂-N), 61.9 (O-CH₂-triazole), 53.0 (C-2′/2′′), 49.5 (N-CH₂CH₂-N).

3.2.14. Synthesis of diosmin peracetate (24)

Diosmin (23, D3525, 0.05 g, 0.08 mmol) and NaF (0.42 g, 10 mmol) were mixed in acetic anhydride (4 mL) and the mixture was kept under MW irradiation (400W) at 130 ºC for 35 minutes. After cooling, the solution obtained was poured into ice and then extracted with CH₂Cl₂. The organic layer was extracted with NaHCO₃, dried over anhydrous Na₂SO₄ and filtered. Crystallization from MeOH/H₂O furnished 5,3-O-acetyl-2-(3-O-acetyl-4-methoxyphenyl)-7-[2,3,4-tri-O-acetyl-α-L-rhamnopyranosyl-(1→6)-2,3,4-tri-O-acetyl-β-D-
glucopyranosyloxyxochromen-4-one (24) as a yellow solid (0.048 g, 0.051 mmol, 62% yield). mp 135-138 °C; IR (KBr) v\text{max}: 2940, 1754, 1644, 1614, 1514, 1433, 1371, 1289, 982 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 300.13 MHz) δ: 7.72 (1H, dd, J= 2.3, 9.0 Hz, H-6'), 7.56 (1H, d, J= 2.3 Hz, H-2'), 7.08 (1H, d, J= 8.8 Hz, H-5'), 6.96 (1H, d, J= 2.4, H-8), 6.65 (1H, d, J= 2.4, H-6), 6.51 (1H, s, H-3), 5.37-5.15 (6H, m, H-1", H-2", H-3", H-4", H-3", H-4''), 5.05-4.97 (1H, m, H-2''), 4.72 (1H, s, H-1'''), 4.01-3.95 (1H, m, H-5''), 3.91 (3H, s, 4'-OCH\textsubscript{3}), 3.85-3.80 (2H, m, H-5'', 6''a), 3.70-3.65 (1H, m, H-6''b), 2.43 (3H, s, COCH\textsubscript{3}), 2.36 (3H, s, COCH\textsubscript{3}), 2.17 (3H, s, COCH\textsubscript{3}), 2.09-2.02 (16H, m, COCH\textsubscript{3}), 1.92 (3H, s, COCH\textsubscript{3}), 1.15 (3H, d, J=6.2, H-6'''). \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75.47 MHz) δ: 176.2 (C-4'), 170.2 (COCH\textsubscript{3}), 170.0 (COCH\textsubscript{3}), 169.9 (COCH\textsubscript{3}), 169.8 (COCH\textsubscript{3}), 169.7 (COCH\textsubscript{3}), 169.4 (COCH\textsubscript{3}), 169.3 (COCH\textsubscript{3}), 168.8 (COCH\textsubscript{3}), 161.5 (C-7), 159.8 (C-2, C-5), 154.0 (C-4'), 150.7 (C-9), 140.1 (C-3'), 123.9 (C-1'), 121.0 (C-6'), 112.8 (C-5'), 112.5 (C-2'), 109.0 (C-3), 102.4 (C-10), 98.1 (C-6, C-1'''), 97.8 (C-8, C-1''), 73.5 (C-5''), 72.4 (C-3''), 70.9 (C-2''), 70.8 (C-4'', C-4''), 69.3 (C-2''), 68.9 (C-3''), 68.6 (C-6''), 66.7 (C-5''), 56.2 (4'-OCH\textsubscript{3}), 31.0 (COCH\textsubscript{3}), 29.7 (COCH\textsubscript{3}), 21.1 (COCH\textsubscript{3}), 20.8 (COCH\textsubscript{3}), 20.8 (COCH\textsubscript{3}), 20.7 (COCH\textsubscript{3}), 20.6 (COCH\textsubscript{3}), 17.3 (C-6'').

3.2.15. Synthesis of rutin peracetate (26)

Rutin (25, R2303, 0.3 g, 0.5 mmol) was mixed with acetic anhydride (12 mL) and the mixture was heated at 130°C until completion of the reaction confirmed by TLC. The solution obtained was poured into ice and extracted with CH\textsubscript{2}Cl\textsubscript{2}. The organic layer was extracted with a saturated solution of NaHCO\textsubscript{3}, dried with anhydrous Na\textsubscript{2}SO\textsubscript{4}, filtered, and then evaporated under reduced pressure. The obtained oil was dissolved in ethyl acetate and insolubilized with petroleum ether 60-80°C and 2-(3,4-di-O-acetylsphenyl)-5,7-di-O-acetyl-3-[3,4,5-tri-O-acetyl-\alpha-L-rhamnopyranosyl-(1→6)-3,4,5-tri-O-acetyl-\beta-D-glucopyranosyloxy]-4H-chromen-4-one (26) was obtained as a white solid (0.43 g, 0.41 mmol, 73% yield). mp 119-120 °C (petroleum ether 60-80°C); IR (KBr) v\text{max}: 2940, 1781, 1754, 1728, 1505, 1477, 1435, 1371, 1145 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 300.13 MHz) δ: 7.96 (1H, dd, J=2.2 and 8.6 Hz, H-6'), 7.91 (1H, d, J=2.1 Hz, H-2'), 7.34 (1H, d, J=8.6 Hz, H-5'), 7.31 (1H, d, J=2.2 Hz, H-8), 6.83 (1H, d, J=2.2 Hz, H-6), 5.43 (1H, d, J=7.8 Hz, H-1''), 5.28 (1H, t, J=9.6 Hz, H-3''), 5.17 (1H, dd, J=7.8 and 9.8 Hz, H-2''), 5.09-5.06 (2H, m, H-2'', H-3''), 4.95 (2H, t, J=9.7 Hz, H-4'', 4''), 4.52 (1H, brs, H-1''), 3.67-3.62 (1H, m, H-5'''), 3.60-3.54 (1H, m, H-5''), 3.53 (1H, dd, J=3.1 and 12.5 Hz, H-6a), 3.26 (1H, dd, J=5.9 and 11.3 Hz, H-6'b), 2.44 (3H, s, COCH\textsubscript{3}), 2.35 (3H, s, COCH\textsubscript{3}), 2.34 (3H, s, COCH\textsubscript{3}), 2.30 (3H, s, COCH\textsubscript{3}), 2.14 (3H, s, COCH\textsubscript{3}), 2.09 (3H, s, COCH\textsubscript{3}), 2.03 (6H, s, COCH\textsubscript{3}), 1.96 (3H, s, COCH\textsubscript{3}), 1.94 (3H, s, COCH\textsubscript{3}), 1.60 (3H, s, COCH\textsubscript{3}), 1.06 (3H, d, J=6.2 Hz, H-6''). \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75.47 MHz)
δ: 171.9 (C-4), 170.2 (COCH₃), 169.9 (COCH₃), 169.9 (COCH₃), 169.8 (COCH₃), 169.8 (COCH₃), 169.6 (COCH₃), 169.3 (COCH₃), 168.1 (COCH₃), 167.9 (COCH₃), 167.8 (COCH₃), 156.6 (C-2), 154.7 (C9), 153.9 (C-7), 150.2 (C-5), 144.1 (C-4'), 141.8 (C-3'), 136.9 (C-3), 128.6 (C-1'), 127.2 (C-6'), 124.7 (C-2'), 123.5 (C-5'), 115.1 (C-10), 113.4 (C-6), 109.1 (C-8), 99.6 (C-1''), 97.7 (C-1'''), 72.8 (C-5''), 72.5 (C-3''), 71.4 (C-2''), 70.9 (C-4''), 69.5 (C-4''), 69.3 (C-2'''), 69.0 (C-3'''), 66.9 (C-6''), 66.3 (C-5'''), 21.2 (COCH₃), 21.1 (COCH₃), 20.9 (COCH₃), 20.7 (COCH₃), 20.7 (COCH₃), 20.7 (COCH₃), 20.6 (COCH₃), 17.2 (C-6''').

3.2.16. Synthesis of naringin persulfate (27)

Naringin (8, N1376, 0.5 g, 0.86 mmol) was dissolved in DMA (10 mL) and SO₃:TEA adduct (9.37 g; 51.6 mmol; 6 eq./OH) was added. The mixture was kept under MW irradiation (200W) at 100°C for 1 hour. After cooling, TEA was added until the pH was 8 and the obtained solution was poured into acetone and left at 4°C for 24 hours. The crude oil formed was washed with acetone and ether to remove unreacted adduct and then dissolved in aqueous solution of 30% NaOAc (4 mL). EtOH was added and the sodium salt of 7-[2-O-(3,4,6-tri-O-sulfate-6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,3-di-O-sulfate-5-O-sulfate-2-(4-O-sulfatepHENYL)-4H-1-benzopyran-4-one (27) insolubilized as yellow solid that was further purified by dialysis using a Spectra/Por 6 regenerated cellulose MWCO 1000 membrane (0.579 g; 0.41 mmol; 48% yield). mp 218-222 °C (H₂O); IR (KBr) vₘₐₓ: 1637, 1518, 1254, 1052, 810; ¹H NMR (DMSO-d₆, 300.13 MHz) δ: 7.58 (2H, d, J=8.8 Hz, H-2',6'), 7.17 (2H, d, J=8.7 Hz, H-3', 5'), 6.20-6.18 (1H, brd, H-6), 6.04-5.95 (1H, m, H-8), 5.31-5.23 (2H, m, H-1'', 1'''), 4.71-4.69 (1H, brd, H-2), 4.56 (1H, d, J=3.1 Hz, H-neohesperidoside), 4.48 (1H, m, H-neohesperidoside) 4.29-4.21 (2H, m, H-neohesperidoside), 4.07-3.94 (3H, m, H-neohesperidoside), 3.82-3.71 (3H, m, H-neohesperidoside), 3.38-3.13 (2H, m, H-3, under water), 1.27 (3H, d, J=6.1 Hz, 6'''-CH₃); ¹³C NMR (DMSO-d₆, 75.47 MHz) δ: 197.3 (C-4), 161.3 (C-7, C-9), 157.0 (C-5), 155.6 (C-4'), 129.8 (C-2', C-6'), 129.5 (C-1'), 120.1 (C-3', C-5'), 101.7 (C-10), 97.5 (C-6), 96.1 (C-1''), 96.0 (C-8), 95.5 (C-1'''), 95.2 (C-8), 79.0 (C-2), 76.9-75.3 (3 C-neohesperidoside), 75.0 (C-4'''), 74.3-67.6 (4 C-neohesperidoside), 67.0 (C-5'''), 40.0 (C-3, under DMSO), 18.1 (C-6'''). HRMS (ESI⁺) m/z calcd for C₂₇H₂₅O₃₈S₉Na₈ 1418.6785, found 1418.67605.
3.2. BIOLOGICAL ACTIVITY

3.2.1. Anticoagulant activity

Clotting times were performed in Laboratório de Medicina Laboratorial Dr Carlos da Silva Torres, Grupo Unilabs, with the technical support of Vítor Marques. Human blood was collected from 7 healthy donors aged between 22 and 30 years old without history of bleeding or thrombosis and who had not taken any medication known to affect blood coagulation and platelet function for 2 weeks. Venous blood was obtained and transferred to a plastic tube. Nine volumes of blood were decalcified with one volume of 3.8% sodium citrate solution. Blood was centrifuged for 20 min at 2400g and the pooled plasma was stored in aliquots at -20 °C until use. Sulfated compounds were dissolved in saline solution. The final concentration of sulfated compounds in these assays ranged from $1.0 \times 10^{-3}$ to $2.56 \times 10^{-5}$ M. Saline was used as control.

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), and APTT assay reagent (50 μL) and the mixture was incubated for 6 min at 37°C. CaCl$_2$ (50 μL, 25 mmol/L) was added, and clotting times were recorded during 180 s. The APTT reagent used was the synthetic phospholipid cephaline with extract of rabbit brain tissue, and a polyphenolic activator.

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. Then PT assay reagent (100 μL) was added and clotting times were recorded during 120 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 (100 μL) was incubated for 2 min at 37°C. Then 100 μL of thrombin solution (100 U/mL), was added and clotting times were recorded during 240 s. The TT reagent used was 100 U/mL of human calcic thrombin.

The clotting times were recorded in seconds (s). Independent experiences were performed. 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 240 s (TT), 180 s (APTT), and 120 s (PT), values of 240, 180, and 120 s, respectively, were arbitrarily assigned for statistical analysis.
3.2.2. Statistical analysis

Statistical significance of the difference between control and treated samples was calculated by unpaired t test using GraphPad Prism 6 software. A value of $P < 0.05$ was considered significant.
CHAPTER 4 - CONCLUSIONS
Considering the general objectives of this work, the synthesis of new anticoagulant analogues of polysulfated small-molecules with improved oral bioavailability, the main achievements are as following:

- sixteen compounds were obtained by different synthetic methods and ten were characterized for the first time;
- copper(I)-catalyzed alkyne-azide 1,4-cycloaddition and sulfation syntheses were successfully assisted with MW irradiation;
- screening of antitumor activity of some synthetic intermediates revealed a promising hit compound, peracetylated derivative 26, that deserves further investigation;
- persulfated conjugate of naringin with bile acid 16 and persulfated xanthone triazole-linked glycoside 22 exhibited anticoagulant activity; persulfated naringin-bile acid conjugate 16 was the most potent anticoagulant compound synthesized in LQOF.

The conjugate of naringin with the bile acid DOCA 16 and the xanthone triazole-linked to a glycoside 22 are expected to cross the GI tract membrane: conjugate 16 will be recognized by bile acid transporters and the triazole in compound 22 is expected to increase lipophilicity.

Taking in account the results obtained in this dissertation, the study of the permeability of the synthesized sulfated compounds would be important to perform.

Improving permeability of polysulfated small-molecules could be a step forward to the development of orally active heparin-like antithrombotic drugs.
CHAPTER 5 - REFERENCES


133. Plaza, C.; Pavani, M.; Araya-Maturana, R.; Pezoa, J.; Maya, J. D.; Morello, A.; Becker, M. I.; De Ioannes, A.; Ferreira, J., Chemosensitizing Effect of Nordihydroguaiaretic
Acid and its Tetra-acetylated Derivative on Parental and Multiresistant TA3 Mouse Mammary Adenocarcinoma Cells. *In Vivo* 2009, 23 (6), 959-967.


CHAPTER 6 – APPENDICES
APPENDIX I - $^1$H and $^{13}$C NMR data for compounds 5 (DMSO-$_d_6$), 6 (CDCl$_3$), and 7 (CDCl$_3$).

The NMR data of compound 5 is in accordance with the literature.\textsuperscript{79} Values of chemical shift ($\delta$) in parts per million (ppm). J values (Hz) are presented in parentheses.

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-OCOCH$_3$ | - | - | 2.53 (s) | 2.49 (s) | 2.34 (s) | 2.32 (s) | 2.07 (s) | 2.06 (s) | 2.03 (s) | 1.80 (s) | 170.6, 170.5 | 170.3, 169.6 | 168.5, 168.0 | 167.7, 167.2 | 21.4, 21.3 | 21.1, 20.8 | 20.7, 20.5 | 20.4, 20.3 | 2.44 (s) | 2.35 (s) | 2.34 (s) | 2.07 (s) | 2.05 (s) | 2.02 (s) | 1.78 (s) | 170.5, 170.2 | 169.5, 169.4 | 169.2, 168.0 | 167.1, 22.7, 21.3 | 20.7, 20.7 | 20.6, 20.5 | 20.4 |

1-OH | 13.77 (s) | - | - | - | 13.25 (s) | - |

3-OH | 10.57 (brs) | - | - | - | - |

6-OH | 4.52 (s) | - | - | - | - |

7-OH | 4.52 (s) | - | - | - | - |
**APPENDIX II** - \(^1\)H and \(^{13}\)C NMR data for compounds 8, 27, 11, and 12 (DMSO-\(d_6\)).

Compound 8 was elucidated in accordance with the literature. Values of chemical shift (\(\delta\)) in parts per million (ppm). J-values (Hz) are presented in parentheses.

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\text{8} & 5.55-5.46 (m) & 78.9 & 4.71-4.69 (m) & 79.0 & 5.60-5.52 (m) & 79.2 & 5.61-5.53 (m) & 79.2 \\
\text{27} & 3.80-3.15 (m) & - & 3.80-3.15 (m) & - & 4.05-3.05 (m) & - & 4.15-3.05 (m) & - \\
\text{11} & 2.78-2.67 (2H, m) & 42.0 & 3.38-3.13 (2H, m)* & - & 2.82-2.73 (2H, m) & 45.4 & 2.82-2.71 (2H, m) & 42.0 \\
\text{12} & 1.25 (OH, s) & 162.9 & - & 1.25 (OH, s) & - & 1.25 (OH, s) & - & 1.25 (OH, s) & - \\
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Potential Under DMSO signal; *under water signal.
APPENDIX III - $^1$H and $^{13}$C NMR data for compounds 9 (DMSO-$d_6$), 13 (CDCl$_3$), and 14 (DMSO-$d_6$).

Compound 9 was elucidated in accordance with the literature.$^{139}$ Values of chemical shift (δ) in parts per million (ppm). J values (Hz) are presented in parentheses.

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APPENDIX IV - ¹H and ¹³C NMR data for compounds 10, 15, and 16 (DMSO-d₆).

Values of chemical shift (δ) in parts per million (ppm). J values (Hz) are presented in parentheses.

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*under water signal
APPENDIX V - \(^1\)H and \(^{13}\)C NMR data for compounds 19, 20, 21, and 22 (DMSO-\(d_6\)).

For all the compounds sugar protons were assigned using the \(^1\)H and \(^{13}\)C data of glucosamine. \(^{140}\) Values of chemical shift (\(\delta\)) in part per million (ppm). \(J\) values (Hz) are presented in parentheses.

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*under water
APPENDIX VI - $^1$H and $^{13}$C NMR data for compounds 23 (DMSO-d$_6$) and 24 (CDCl$_3$).

Data for compound 23 is in accordance with the literature. Values of chemical shift ($\delta$) in parts per million (ppm). $J$ values (Hz) are presented in parentheses.

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-COCH$_3$ | - | 2.43 (s) | 2.36 (s) | 2.17 (s) | 2.09-2.02 (m) | 1.92 (s) | 170.2, 170.0 | 169.9, 169.8 | 169.7, 169.4 | 169.3, 168.8 | 51.0, 29.7 | 21.1, 20.8 | 20.8, 20.7, 20.6 |
APPENDIX VII - $^1$H and $^{13}$C NMR data for compounds 25 (DMSO-$d_6$) and 26 (CDCl$_3$).

Compound 25 was elucidated in accordance with the literature.$^{74, 129}$ Values of chemical shift (δ) in parts per million (ppm), J values (Hz) are presented in parentheses.

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