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EFFECT OF HYDROXYLATED POLYMETHOXYFLAVONES AND
ACETYLATED POLYMETHOXYFLAVONES ON ADIPOGENESIS *IN VIVO* AND

IN VITRO

By

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A dissertation submitted to the
Graduate School-New Brunswick
Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Food Science

Written under the direction of

Professor Chi-Tang Ho

And approved by

New Brunswick, New Jersey

October, 2015

ABSTRACT OF DISSERTATION
EFFECT OF HYDROXYLATED POLYMETHOXYFLAVONES AND
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Excess energy is a major cause of obesity and it can increase the number and size of adipocytes, eventually expanding the adipose tissue. Adipose tissue can be deposited in the intra-abdominal area and it affects the function of other organs, such as the liver, pancreas, and skeletal muscle, therefore, it is a risk factor related to liver disease and type 2 diabetes. Diet modification and the inhibition of the cause of obesity-related molecular mechanisms could be solutions for controlling the expansion of adipose mass and decreasing the prevalence of obesity. Polymethoxyflavones (PMFs) and hydroxylated polymethoxyflavones (HPMFs) such as nobiletin, tangeretin, 5-demethyltangeretin, and 5-demethylnobiletin are unique flavonoids that almost exclusively exist in the peel of the citrus genus and have many health-beneficial effects. However, their lipophilic structure and characteristics give them poor aqueous solubility and low oral bioavailability.

In the current research, we prepared 5-demethyltangeretin (5-OH-Tan), 5-demethylnobiletin (5-OH-Nob), 5-acetyloxy-6,7,8,4'-tetramethoxyflavone (5-Ac-Tan),

and 5-acetyloxy-6,7,8,3',4'-pentamethoxyflavone (5-Ac-Nob) through chemical modification from nobiletin and tangeretin. Firstly, we found that 5-Ac-Nob had better anti-adipogenesis activity than 5-OH-Nob, 5-OH-Tan, and its anti-adipogenesis ability by increased the phosphorylated-LKB1 and AMPK α protein levels and decreases the transcriptional factor SREBP-1 and lipogenesis-related enzyme fatty acid synthase protein levels in a 3T3-L1 preadipocyte model. Therefore, we further investigated the anti-adipogenesis effect of 5-Ac-Nob by using a diet with 45% calories from fat to induce obesity in C57BL/6J male mice. We found that mice fed with 5-Ac-Nob had lower body weight; intra-abdominal fat, plasma and liver triacylglycerol levels, and plasma cholesterol level and it had potential to prevent fatty liver by increased phosphorylated-LKB1 and AMPK α protein levels and increased the level of a lipogenesis-related enzyme, an inactive form of phosphorylated acetyl-CoA carboxylase protein, in the liver. In addition, we also found that 5-OH-Nob could be a metabolite hydrolyzed from 5-Ac-Nob in plasma when the mouse were administered with 100 mg/kg bw of 5-Ac-Nob by oral gavage. All these results showed that 5-Ac-Nob could be a 5-OH-Nob prodrug to alleviate lipid accumulation by activated AMPK α and then affect lipid synthesis *in vivo* and *in vitro*.

Acknowledgements

I truly appreciate Dr. Chi-Tang Ho serving as my advisor. He gave me tremendous guidance during my graduate studies and research.

I also thank my committee members, Dr. Thomas G. Hartman, Dr. Qingrong Huang, and Dr. Shiming Li for their time and great suggestions regarding this dissertation.

I offer special thanks to Dr. Guor Jien Wei for his kind provision of compounds for this dissertation and to Dr. Min-Hsiung Pan for his great suggestions and guidance for the dissertation.

I thank my friends Wei-Lun Hung, Pei-Hsuan Hsieh, Hui-Yun Tsai, Tzi-Min Wang, and Chen-Yu Tsai and my lab mates at National Taiwan University and National Kaohsiung Marine University for their kind help.

I greatly appreciate my parents, my sister, and my brother for their great support in making my dream come true.

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ABBREVIATIONS

5-OH-Tan: 5-demethyltangeretin

5-OH-Nob: 5-demethylnobiletin

5-Ac-Tan: 5-acetyloxy-6,7,8,4'-tetramethoxyflavone

5-Ac-Nob: 5-acetyloxy-6,7,8,3',4'-pentamethoxyflavone

ACC: Acetyl-CoA carboxylase

AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase

BMI: Body mass index

CDC: Centers for Disease Control and Prevention

C/EBPs: CCAAT-enhancer-binding proteins

DEX: Dexamethasone

ECM: Extracellular matrix

FAS: Fatty acid synthase

HFD: High-fat diet

HPMFs: Hydroxylated polymethoxyflavones

IBMX: 3-Isobutyl-1-methylxanthine

IGF-1: Insulin-like growth factor 1

LKB1: Liver kinase B1

MCE: Mitotic clonal expansion

NIH: National Institutes of Health

pACC: Phosphorylate acetyl-CoA carboxylase

PMFs: Polymethoxyflavones

PPAR: Peroxisome proliferator-activated receptor

SREBPs: Sterol regulatory element binding proteins

TG: Triacylglycerol

WHO: World Health Organization

CHAPTER 1. INTRODUCTION

The increasing prevalence of obesity is happening not only in the United States but also worldwide. According to WHO data, the prevalence of obesity has doubled since 1980. Almost 65% of the world's population lives in countries where overweight and obesity kills more people than underweight.¹ Today, obesity affects not only adults but also children; 15–20% of children and adolescents are obese in the United States.² Obesity is a serious health problem worldwide. The primary cause of obesity is an energy imbalance. When energy intake is more than energy expenditure, the excess energy could be stored as triacylglycerol in the adipocytes. This excess energy can increase the number and size of adipocytes, eventually expanding the adipose tissue. Adipose tissue can be deposited under the skin and in the intra-abdominal area. More and more research has shown that adipose tissue is an endocrine organ that can regulate the whole-body energy homeostasis through the secretion of various adipose-derived hormones or adipokines and affects the function of other organs, such as the brain, liver, pancreas, and skeletal muscle.³ Therefore, obesity plays a key role in metabolic syndromes and is highly related to diabetes, cardiovascular disease, and cancer.^{4, 5} The National Institutes of Health has developed some strategic plans for obesity research, including the discovery of biological mechanisms regulated to energy balance; understanding of the correlates, determinants,

and consequences of obesity; the design and testing of interventions to promote healthy weight; the dissemination and implementation of research; and improvements in measurement tools, technology, and methods. Their ultimate goal is to prevent and treat obesity efficiently.⁶ Recently, researchers have been more interested in the role of natural compounds such as epigallocatechin-3-gallate from green tea, raspberry ketones from raspberries, and synephrine from orange in regulating lipid metabolism to decrease adipogenesis *in vivo* or *in vitro*.⁷⁻⁹ Polymethoxyflavones (PMFs) and hydroxylated polymethoxyflavones (HPMFs) such as nobiletin and tangeretin are a type of flavonoid found in citrus fruits and they can suppress lipid accumulation in 3T3-L1 preadipocytes and in high-fat diet mice models.¹⁰⁻¹² However, both PMFs and HPMFs are more lipophilic structure and characteristics give them poor aqueous solubility and low oral bioavailability. Therefore, in the current study, we based on the prodrug concept like aspirin (acetylsalicylic acid) as a prodrug of salicylic acid that make less quantity of salicylic acid more efficiency and more tolerance in the body.¹³ We used chemical modification to obtain 5-demethylated tangeretin (5-OH-Tan), 5-demethylated nobiletin (5-OH-Nob), and 5-acetyloxy-6,7,8,4'-tetramethoxyflavone (5-Ac-Tan), 5-acetyloxy-6,7,8,3',4'-pentamethoxyflavone (5-Ac-Nob) from tangeretin and nobiletin, individually and investigated their anti-adipogenesis activity in a 3T3-L1 preadipocyte model and in

the high-fat diet-induced obesity animal model. In addition, we also try to understand the absorption of 5-OH-Nob and 5-Ac-Nob in mouse plasma.

CHAPTER 2. LITERATURE REVIEW

2.1 Epidemiology of obesity

Obesity is the second leading cause of preventable death in the United States. The WHO estimates the prevalence of overweight adults to be as high as 2.3 billion and that of obese adults to be 700 million in 2015.¹⁴ The estimated health care costs of obesity are up to 8% of total health care costs in Western countries, and the annual extra medical costs of obesity were around \$75 billion in the United States.¹⁵⁻¹⁷ Overweight and obesity in adults are defined by the World Health Organization (WHO) and the National Institutes of Health (NIH) on a simple formulation, body mass index (BMI), which is determined as weight (kg) divided by height² (m). A BMI value from 25–29.9 kg/m² indicates overweight, and obesity is a BMI ≥ 30 kg/m.^{18, 19}

Table 1. WHO Standard Classification of Obesity¹⁷

| WHO Standard Classification of Obesity | | |
|--|--------------------------|-----------------------|
| | BMI (kg/m ²) | Risk of Comorbidities |
| Normal range | 18.5-24.9 | Average |
| Overweight | 25-29.9 | Mild increase |
| Obesity class I | 30.0-34.9 | Moderate |
| Obesity class II | 35-39.9 | Severe |
| Obesity class III | ≥ 40 | Very severe |

Source: WHO, Obesity: Preventing and Managing the Global Epidemic, Report of a WHO Consultation on Obesity,

Technical Report Series, No.894, World Health Organization, Geneva, Switzerland, 2000, p. 256.

The childhood (age birth to 19 years) obesity epidemic is in rising, according to Centers for Disease Control and Prevention (CDC) growth charts, and is defined by weight at or above the 95th percentile for age. Based on these definitions, the prevalence of childhood obesity in the United States has tripled in just 30 years. The increased incidence of childhood obesity is related to increased rates of hyperlipidemia and type 2 diabetes mellitus in children.¹⁹⁻²¹ The obesity prevalence rates increased in all gender-ethnic groups with time and in all income and educational levels.^{2, 22, 23} BMI is a simple method for evaluating obesity; however, the distribution of body fat is another important indicator of obesity. Visceral (intra-abdominal) obesity, in contrast to subcutaneous (lower-body) obesity, is the greatest risk factor for chronic-degenerative diseases, including heart disease and type 2 diabetes mellitus, and it can be determined by waist circumference.¹⁷

Table 2. Waist Circumference¹⁷

| Sex-Specific Waist Circumferences for Increased Risk and Substantially Increased Risk of Metabolic Composition Associated with Obesity in Caucasians | | |
|--|-----------|-------------------------|
| | Increased | Substantially Increased |
| Men | ≥94 cm | ≥102 cm |
| Women | ≥80 cm | ≥88 cm |

Source: WHO, Obesity: Preventing and Managing the Global Epidemic, Report of a WHO Consultation on Obesity, Technical Report Series, No.894, World Health Organization, Geneva, Switzerland, 2000, p. 256.

For example, a person who has a low BMI value but high levels of abdominal fat is at high risk of developing type 2 diabetes and heart disease.¹⁷ Recently, a new term was

coined, “diabesity,” to refer to diabetes in the context of obesity or obesity-dependent diabetes and it has become an epidemic.^{24, 25} Overall, obesity causes adverse effects from childhood to adulthood and costs a great deal of money for medical expenditures.

2.2 Cause of obesity

Many factors influence the development of obesity, such as the methodological limitations to accurately measure people’s dietary energy intake and energy expenditure.²⁶ Genes cause variation and susceptibility among different races and ethnicities.²⁷ Low-cost energy-dense foods and limited physical activity are additional environmental factors that could provide more support to illustrate the development of obesity. For example, the consumption of more high-fructose corn syrup and people with a low level of physical activity are more likely to gain around 13 kg in ten years are environment factors of the development of obesity.^{2, 7, 28} The cause of obesity is very complex, but it can simply be based on an imbalance between energy intake and energy expenditure.

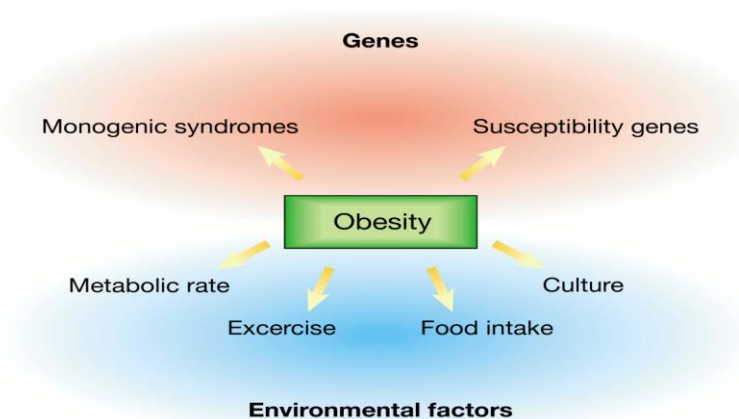


Figure 1. Factors influencing the development of obesity.²⁹

2.3 Management of obesity

Reduced energy intake and increased energy expenditure is the basic principle for managing overweight and obesity. Dietary treatment is a method to reduce energy intake and its major characteristics are low fat, particularly saturated fat, increased whole-grain/high-fiber carbohydrates, increased intake of fruit and vegetables, and proper portion sizes such as low-calorie diets (800–1,500 kcal/day), low-fat/high-carbohydrate diets, and very low-calorie diets (450–800 kcal/day); low glycemic index diets and high-protein/low-carbohydrate diets have also been proposed³⁰. More physical activity could increase energy expenditure like thirty minutes of moderate activity at least five days a week could improve fitness and protect against cardiovascular diseases and 45–60 minutes of daily activity could maintain weight and prevent weight regain.^{30, 31} Drugs are another anti-obesity option; they affect the gastrointestinal system and the central nervous

system to suppress appetite. Surgical treatments such as gastric restriction and gastric bypass operations are used to address morbid obesity. Drugs and surgical treatment are not suitable for all obese people; they must be evaluated by doctors and require intensive care.³⁰ Diet treatment and physical activity are suitable not only for obese people but also for everyone who wants to maintain his/her health.

2.4 Adipose tissue and adipocytes

There are two kinds of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). The most predominant characteristic of BAT is non-shivering thermogenesis, where energy derived from fatty acid oxidation generates heat by mitochondrial uncoupling to maintain body temperature.^{3, 32, 33} WAT is a fuel storage organ; it stores excess energy as triacylglycerol by a lipogenesis process and uses triacylglycerol during food deprivation by a lipolysis process.^{3, 34} WAT not only plays a key role in energy homeostasis, but also in insulin signaling, and endocrine action and acts like an endocrine organ, releasing a wide range of adipokines and regulating immune responses, blood pressure control, angiogenesis, hemostasis, bone mass, and thyroid and reproductive function.^{35, 36} Adipose tissue contains several cell types, including endothelial cells, blood cells, fibroblasts, preadipocytes, macrophages, and other immune

cells, and mature adipocytes are the most abundant.^{35, 37} Hyperplasia (an increase in adipocyte number) and hypertrophy (an increase in adipocyte volume) are two important characteristics of obesity. Adipocyte hyperplasia can increase adipose tissue mass by increasing numbers of preadipocytes and the differentiation of preadipocytes into mature adipocytes.³⁸ Adipocyte hypertrophy is one mechanism for increasing adipose tissue in adults and has been shown in overweight and type 2 diabetes patients.^{3, 38, 39} When adipocytes increase in large numbers, they become the main cell type and then form adipose tissue. All these phenomena show that an increase in the number and size of adipocytes causes WAT to expand and leads to obesity.^{35, 40}

2.5 Distribution of adipose tissue

The different regions of the adipose tissue deposits show different structural organizations, cellular size, and biological function.⁴¹ Research has shown that intra-abdominal adipose tissue (visceral fat) that WAT surrounds the organs poses a higher risk factor associated with type 2 diabetes and cardiovascular diseases than subcutaneous tissue that WAT under the skin.^{3, 42-44} In severe obesity, the adipocytes are unable to store excess lipid and even to enhance adipocyte proliferation, and lipids can accumulate in non-adipose tissue such as the liver, skeletal muscle, and the heart and the fat around organs may impair their function.^{35, 42} In extremely severe obesity, adipocytes

will lose their function and increase lipolysis, which then increases plasma free fatty acid especially intra-abdominal adipose tissue can more easily transport free fatty acid into the liver by portal circulation than subcutaneous adipose tissue and TG synthesis occur in the liver^{42, 45-48} cause fatty livers and impaired liver function, which may lead to nonalcoholic fatty liver diseases.^{42, 49} In the figure 2, retroperitoneal, perirenal, mesenteric and perigonadal are intra-abdominal fats in mice.³

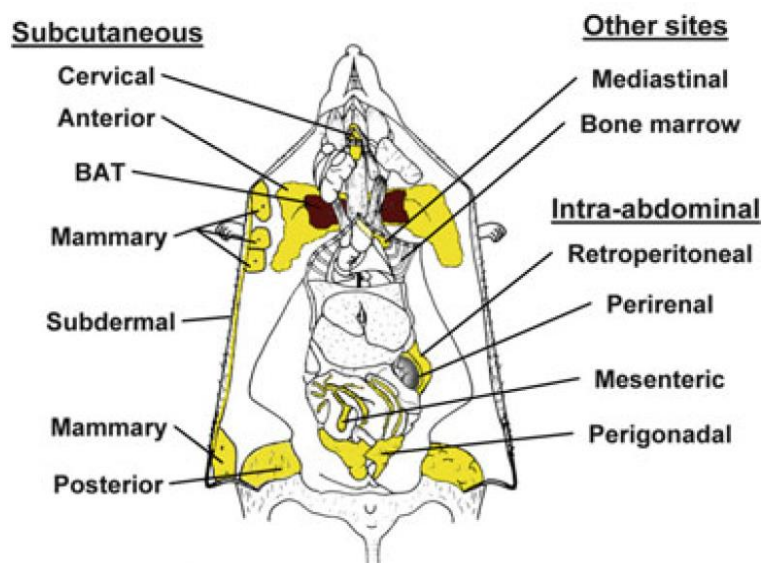


Figure 2. White adipose tissue distribution in mice.³

2.6 Adipogenesis – The stage of cell differentiation

Adipogenesis is a process by which fibroblast-like preadipocytes differentiate into mature adipocytes and there are large internal fat droplets in mature adipocytes.^{35, 50} The process of adipogenesis involves six stages: mesenchymal precursors, committed preadipocytes, growth-arrested preadipocytes, mitotic clonal expansion, terminal differentiation, and

mature adipocytes.⁵⁰ Therefore, in process of adipogenesis a series of cell-cycle protein, transcription factors, lipogenesis related gene and enzyme activity are involved.

3

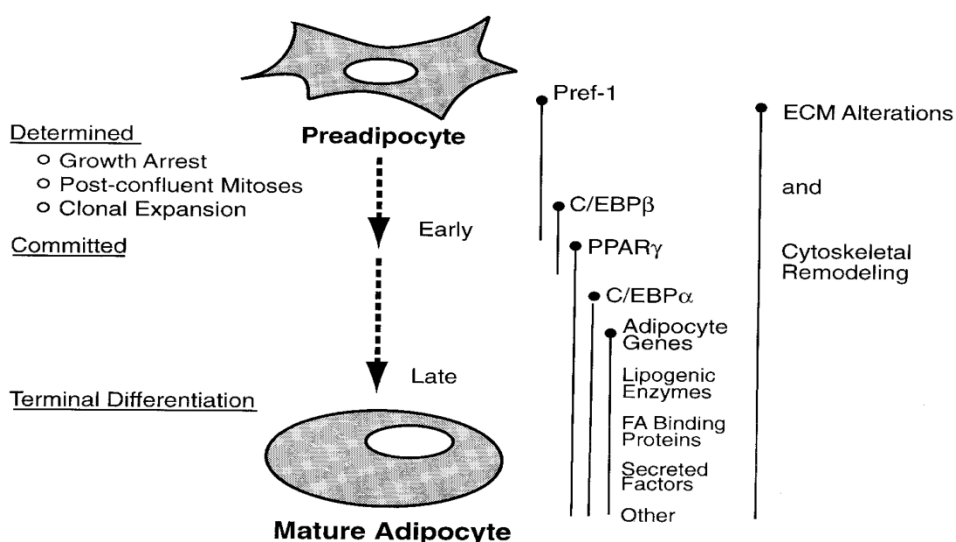


Figure 3. Overview of stages in adipocyte differentiation.⁵¹

The cell shape will change dramatically from fibroblastic to spherical through the level and type of extracellular matrix (ECM), and the level of cytoskeletal components changes in the early stage of adipogenesis (Figure 3).⁵¹ Growth arrest is another important process that occurs during adipocyte differentiation in both preadipocyte cell lines and primary preadipocytes, regulated by transcriptional factors CCAAT-enhancer-binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor (PPAR) families, such as C/EBP α and PPAR γ .⁵² After cell cycle arrest, the cells re-enter the cell cycle and

undergo cell divisions through a mitotic clonal expansion (MCE) period.⁵³ When adipocytes re-enter the cell cycle, the cells will then move to the late stage of adipogenesis, the activation of the transcriptional cascade will increase, protein and mRNA levels of triacylglycerol synthesis and lipogenesis related enzymes such as ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase, glycerol-3-phosphate dehydrogenase, fatty acid synthase (FAS), and glyceraldehyde-3-phosphate dehydrogenase protein expression or enzyme activity.^{3, 54} Therefore, there are fat droplets in the mature adipocytes.

2.7 Adipogenesis-transcriptional factors

CCAAT-enhancer-binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor (PPAR) families play an essential role in the differentiation process (Figure 3).^{40, 55} The PPAR γ gene has two major protein isoforms, PPAR γ 1 and PPAR γ 2; these two isoforms are expressed in most adipocytes.^{50, 56} C/EBP α , - β , and - δ are important transcription factors in adipocyte differentiation and adipogenesis in mouse models. C/EBP β has maximal expression in early adipogenesis and induces PPAR γ and C/EBP α and it also has similar DNA binding properties to C/EBP α and exhibits transcriptional activity in mature adipocytes.^{33, 57} Sterol regulatory element binding proteins (SREBPs) are another transcription factor related to cellular lipogenesis, lipid homeostasis, and

adipocyte differentiation. They can regulate cholesterol, fatty acid, triacylglycerol, and phospholipid synthesis-related enzyme expression.^{40, 58, 59} Generally, the activation of SREBPs requires a two-step cleavage process; after it has been cleaved, its nuclear form is released from the endoplasmic reticulum membrane and then to the promoters of target genes to activate transcription.⁵⁸⁻⁶⁰ Studies have shown that there are two separate SREBP genes in mammals, SREBP1 and SREBP2. SREBP1 can induce PPAR- γ expression and regulate lipid biosynthesis in adipocytes and increase lipogenic gene expression such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) gene expression.^{3, 61, 63, 64} Therefore, the activities of ATP-citrate lyase, acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) are greatly increased during adipogenesis.^{30, 62-64}

2.8 Adipogenesis-cell model for differentiation

Various cell culture models, including preadipocyte cell lines and primary culture of adipose-derived stromal vascular precursor cells, are used to study the molecular mechanisms of adipocyte differentiation. However, the study of preadipocyte differentiation *in vivo* is very difficult because the components of human and animal fat tissue are combined with small blood vessels, nerve tissue, fibroblasts, and preadipocytes at various stages of development. It is hard to distinguish preadipocytes from fibroblasts because preadipocytes cannot be in the same developmental stage *in vivo*.^{37, 40, 51}

Although primary preadipocyte culture has been used to study adipogenesis, it still has many problems. Contamination, the limited life span, and the low percentage of preadipocytes in the total adipose tissue are big problems for primary preadipocyte cell culture. Therefore, the molecular mechanism of adipogenesis is intensively studied *in vitro* by using different pre-adipocyte clonal cell lines from mice or rats^{40, 53}. The 3T3-L1 cell line is one a well-characterized and reliable model for studying the conversion of preadipocytes into adipocytes, established by Green and Kehinde and derived from disaggregated 17- to 19-day-old Swiss 3T3 mouse embryos.^{51, 65, 6669} 3T3-L1 preadipocyte differentiation has most of the characteristics of adipocytes from animal tissue. More than 5,000 published articles have used the 3T3-L1 cell line model to study adipogenesis and the biochemistry of adipocytes.⁶⁷ When 3T3 lines reach confluence, part of a cell could spontaneously differentiate into rounded cells and contain large droplets of triacylglycerol, and the cells show 10- to 100-fold increases in *de novo* lipogenesis, 40- to 50-fold increases in ATP-citrate lyase, ACC, and FAS activity, and 80- and 15-fold increases in glycerophosphate acyltransferase and malic enzyme, respectively.^{62, 68} However, the adipocyte conversion process can occur spontaneously around two to four weeks after confluence, and the conversion process can be accelerated by an adipogenic cocktail. The adipogenic cocktail or differentiation medium (DM)

includes insulin, a glucocorticoid, an agent that elevates intracellular cAMP levels, and fetal bovine serum and the cells can synthesize cyclic AMP to respond to adrenocorticotrophic hormone and become more sensitive to insulin.⁷² Insulin acts through the insulin-like growth factor 1 (IGF-1) receptor. Dexamethasone (DEX) is a synthetic glucocorticoid agonist used to stimulate the glucocorticoid receptor pathway. 3-Isobutyl-1-methylxanthine (IBMX) is a cAMP-phosphodiesterase inhibitor used to stimulate the cAMP-dependent protein kinase pathway.^{52, 69-71} After the addition of DM, C/EBP β and δ are the first transcription factors after exposure to DM and they will be expressed within 1 h in 3T3-L1 preadipocyte differentiation.⁷² Basically, C/EBP β is responsive to DEX and C/EBP δ is responsive to IBMX in the DM and C/EBP δ expression will disappear in the subsequent 48 h, and C/EBP β declines around day 8 after the removal of DM.^{53, 73, 74} Rosiglitazone is a type of thiazolidinedione compound, and it could activate the important transcriptional factor: PPAR γ and it can increase the small number of adipocytes and fat mass in animal models and human subject.⁷⁵⁻⁷⁷ Zebisch et al. added rosiglitazone to a DM as an additional prodifferentiative agent in 3T3-L1 preadipocytes and achieved complete differentiation within 10 to 12 days.⁷⁸

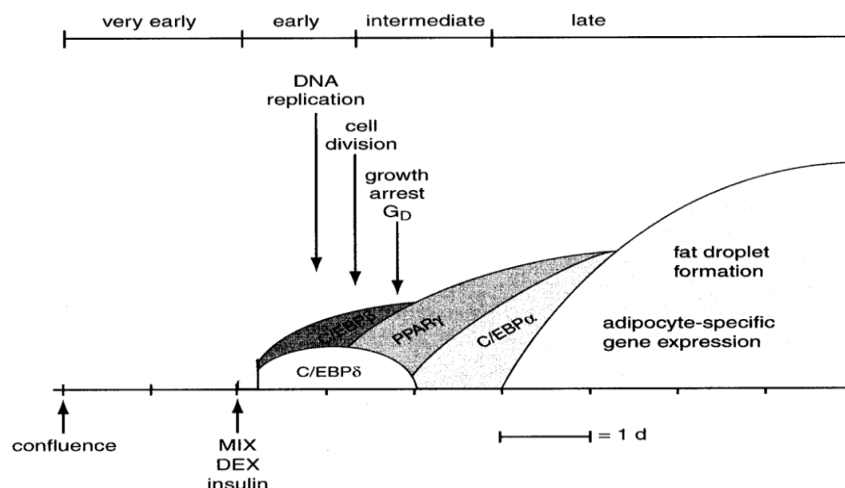


Figure 4. Progression of 3T3-L1 preadipocyte differentiation.⁵⁵

2.9 High-fat diet-induced obesity mice model

Dietary fat is always highly related with increasing adiposity, and when people intake 30% energy from fat in a high-fat diet, it can easily induce obesity in human and in animals, including rats and mice.⁷⁹⁻⁸¹ 30–78% of total energy intake is usually imposed by adding a particular fat to the diet or using a variety of fat- and sugar-rich foods as a cafeteria diet for in rats and mice.⁸²⁻⁸⁵ When rats and mice are fed a high-fat diet, there is a positive relationship between body weight or fat gain and the amount of fat in the diet.^{82, 83, 86-88} Some studies have shown that the dietary fatty acid profile is more important than all the individual fats in developing dietary obesity, but more research is needed to confirm the findings^{83, 89-92}, but studies have shown that lard as a major fat source in an HFD could increase body weight more than olive oil and that coconut oil and fish oil are not good choices for a fat source in an HFD for 11 weeks.^{82, 93} Although, each

individual may have a different genetic background, and different genetics can affect the level of obesity in both human and animal models.⁹⁴ Today, rats and mice are the standard models for studying dietary obesity, and it is easier to induce obesity by an HFD in Sprague–Dawley rats, Wistar rats, and C57BL/6C mice than in other species.⁸² C57BL/6J mice are most frequently used in HFD-induced obesity animal models because HFD diet-induced abnormalities in C57BL/6J mice are almost as the same as those of human metabolic syndrome.^{95, 96,}

2.10 Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK)

Obesity results from an imbalance between energy in and energy out. Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) can increase energy production activity such as glucose transport and fatty acid oxidation and decrease energy consumption activity such as lipogenesis, protein synthesis, and gluconeogenesis.⁹⁷ and it has been studied in muscles, liver, and adipocyte.⁹⁸ AMPK can switch on the catabolic pathway and switch off the anabolic pathway. These effects can occur through different mechanisms in different time frames: 1) acute effects on metabolism related to the phosphorylation of metabolic enzymes, such as the inhibition of cholesterol synthesis by phosphorylating 3-hydroxy-3-methylglutaryl-CoA reductase; 2) longer-term effects that affect in gene expression, such as the upregulation of glucose and fat oxidation by

increasing the expression of mitochondrial genes or down-regulation of gluconeogenic genes; and 3) both acute and longer-term effects, such as the inhibition of fatty acid synthesis by directly phosphorylating the ACC1 isoform of acetyl-CoA carboxylase or along with the inhibition of expression of the ACC1 and fatty acid synthase genes.⁹⁹

AMPK has three subunits: one is a catalytic subunit (α) and the other two are regulatory subunits (β and γ). The α subunit is a catalytic core that contain the threonine 172 residue is responded for phosphorylation, expressed in rat adipose tissue.¹⁰⁰ Liver kinase B1 (LKB1), a upstream of AMPK and it can phosphorylate the threonine residue (Thr-172) of AMPK to activate AMPK in mouse liver, muscle, and adipocyte cell lines to regulate the lipid metabolism.^{101, 102} The LKB1-AMPK signaling pathway is activated by elevation of the AMP/ATP ratio such as glucose deprivation, hypoxia, ischemia exercise and fasting can activate AMPK and then increase lipolysis or inhibit fatty acid and triglyceride synthesis.⁹⁷ Recently, studies have reported that AMPK can directly phosphorylate SREBP (SREBP1c and SREBP2) to suppresses SREBP-1c cleavage and nuclear translocation, then inhibites SREBP-1c target gene expression to reduced lipogenesis and lipid accumulation in hepatocytes with high glucose status.^{9, 103} And SREBP1 also can induce a gene program related to ACC1 and FAS protein epression. Therefore, AMPK can acutely control lipid metabolism by phosphorylating ACC1 and

ACC2 or regulate long-term effects on phosphorylated SREBP1 and the loss of expression of lipogenic enzymes.¹⁰³

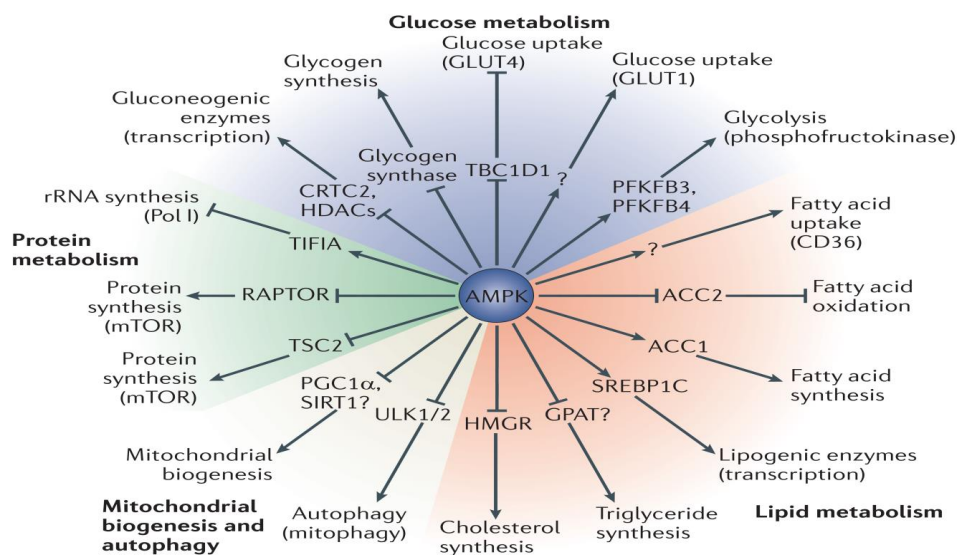


Figure 5. Effects of the activation of AMPK on cellular metabolism.¹⁰³

CHAPTER 3. POLYMETHOXYFLAVONES

3.1 Polymethoxyflavones and structure

Polymethoxyflavones (PMFs) and hydroxylated polymethoxyflavones (HPMFs) are found almost exclusively in the peels of the citrus genus, and they have been used for herbal medicines to treat different diseases. PMF is a general term for flavones that have two or more methoxy groups on their basic benzo- γ -pyrone (15-carbon, C6–C3–C6) skeleton with a carbonyl group at the C4 position.¹⁰⁴ Nobiletin and tangeretin are the two most common PMFs in citrus peels. HPMFs are kind of a PMF with one or two methoxy

groups replaced with hydroxy group(s), such as 5-demethylheptaMF, 5-OH-Tan, and 5-OH-Nob, and they have also been found in citrus peels.¹⁰⁵ 5-hydroxy PMFs can be synthesized by a one-step conversion from PMFs under acidic conditions.¹⁰⁶

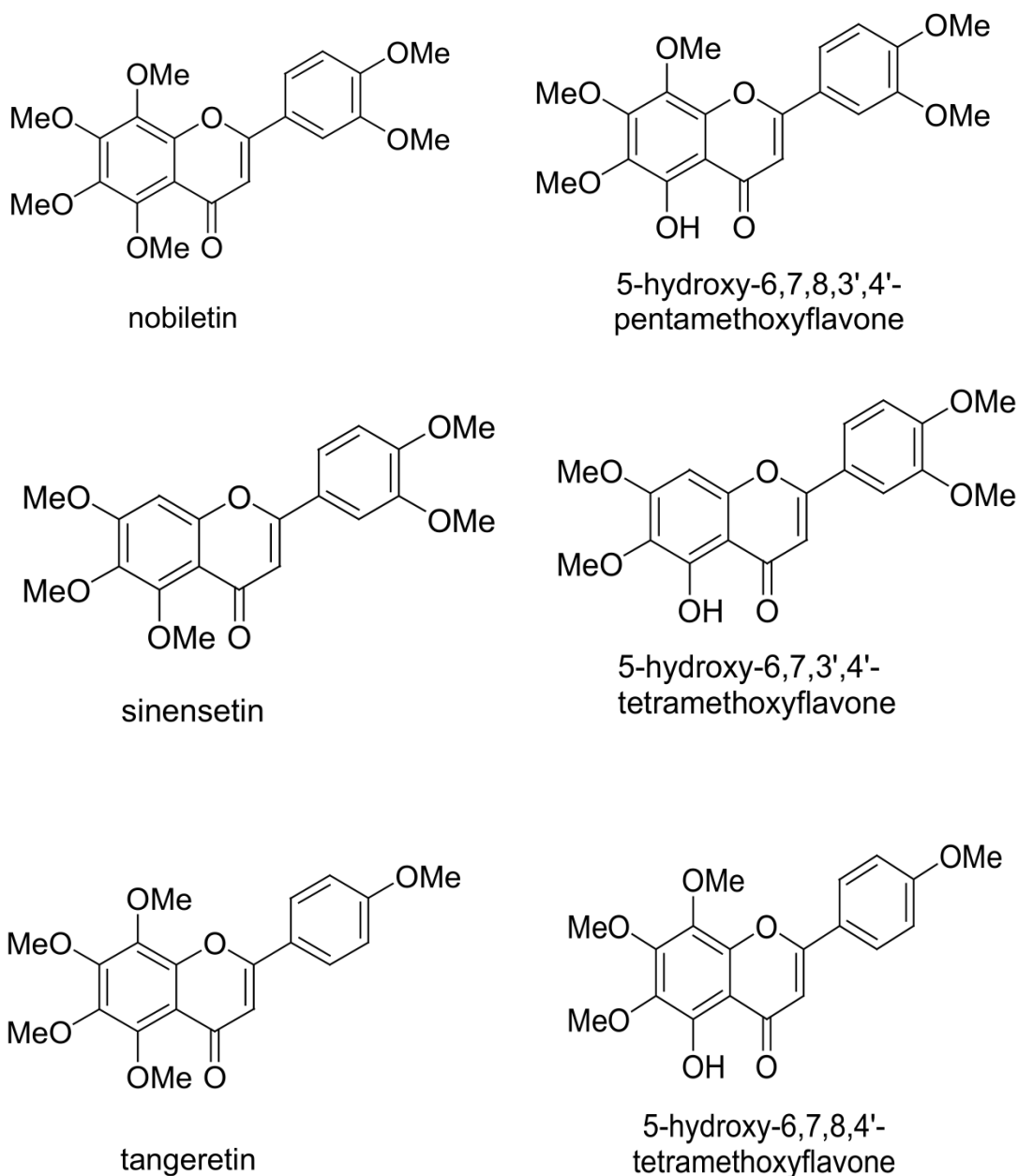


Figure 6. Structure of PMFs and HPMFs.¹⁰⁵

3.2 Biological activities

PMFs exhibit many biological activities, including anti-cancer,^{104, 107} anti-inflammatory,¹⁰⁸ and anti-atherogenic¹⁰⁹ activities and the regulation of lipid homeostasis.^{110, 111} Recent studies have shown that HPMFs exhibit better anti-cancer and anti-inflammatory activities than their related PMFs,^{112, 113} and they can also regulate lipid metabolism.¹⁰¹ Tangeretin and nobiletin are the most abundant PMF in citrus peels; therefore, they have shown anti-cancer^{114, 115} and anti-inflammatory properties and regulate lipid metabolism *in vivo* and *in vitro*.¹¹

3.2.1 Anti-cancer activity

Tangeretin induces apoptosis through p53-dependent mitochondrial dysfunction and the Fas/FasL-mediated extrinsic pathway in human gastric cancer cells.¹¹⁶ Tangeretin can also upregulate the function of the E-cadherin/catenin complex to inhibit invasion in human MCF-7/6 breast carcinoma cells.¹¹⁷ Nobiletin induces cell-cycle arrest at the G0/G1 phase by suppressing ERK1/2 activity, cyclin-D1 and p21 upregulation in human breast cancer.¹¹⁸ Nobiletin suppresses human colorectal cancer cell invasion and metastasis by decreasing the amount of proMMP-7 protein and its mRNA expression in both a concentration- and time-dependent manner.¹¹⁹ Nobiletin, tangeretin, and their related hydroxylated forms can inhibit HL-60 cancer cell proliferation by inducing

apoptosis and 5-OH-Nob, 5-demethylheptaMF, and 5-demethylhexaMF even show stronger anti-cancer activity than their related PMFs.¹⁰⁵

3.2.2 Anti-inflammatory activity

Tangeretin exerts anti-neuroinflammatory effects by inhibiting the LPS-induced phosphorylation of ERK, N-terminal kinase (JNK), and p38 in microglial cells.¹²⁸

5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (5-OH-HxMF) has anti-inflammatory effects through the down-regulation of inflammatory iNOS and COX-2 gene expression in mouse skin antitumor models.¹¹² Nobiletin, its anti-inflammatory effects by significantly inhibiting LPS-induced intracellular ROS production or just like its metabolites (3'- and 4'-demethylnobiletin) and di-demethylation (3',4'-didemethylnobiletin) exhibit anti-inflammatory activity through decreased LPS-induced NO production and iNOS and COX-2 protein expression in RAW264.7 and the metabolites have better anti-inflammatory activity than their parent compound.¹¹³

3.2.3 Lipid metabolism

Citrus flavonoids, including naringenin, hesperidin, nobiletin, and tangeretin, have been studied for the treatment of metabolic dysregulation, for examples, prevent hepatic steatosis, dyslipidemia, and insulin sensitivity by inhibiting hepatic fatty acid synthesis and increasing fatty acid oxidation.¹²⁰ Both nobiletin and tangeretin can suppress

triacylglycerol accumulation in mature 3T3-L1 adipocytes by regulating adipocytokine secretion.¹¹ Nobiletin has also been proved to suppress adipocyte differentiation by downregulating the PPAR γ 2 gene level and decreases the phosphorylated signal transducer and activator of transcription (STAT) 5 protein levels or by blocking the adipocyte transcriptional factor PPAR γ and C/EBP α expression and increases AMPK phosphorylation in 3T3-L1 preadipocyte models^{12, 98} Citrus peel extracts, including nobiletin and tangeretin have been shown to decrease body weight gain in an HFD-induced obesity mice models by decreasing the mRNA levels of lipogenesis-related genes, including stearyl-CoA desaturase, ACC, fatty acid transport protein, and diacylglycerol acyltransferase 1.¹²¹ Recently, HPMFs have been proved to decrease adipogenesis in a 3T3-L1 preadipocyte model by downregulating PPAR γ , SREBP1c, FAS, and ACC protein levels and increase phosphorylated AMPK α protein levels and decrease body weight and intra-abdominal fat in a HFD-induced obesity animal model.¹⁰

3.3 Bioavailability

Bioavailability is affected by absorption, distribution, metabolism, and excretion. However, bioavailability studies of PMFs and HPMFs are rare. Therefore, the bioavailability and absorption of PMFs are based on their structural characteristics. PMFs and HPMFs have many methoxy groups on their skeleton backbone; therefore, they have

more lipophilic properties, poor aqueous solubility, and low bioavailability.¹⁰⁴ According to the results of a lyophilization solubility assay,¹²² the overall solubility of PMFs is very low, but the solubility of HPMFs is better than that of their parent compounds. The solubility of PMFs and HPMFs, from high to low, is as follows: 3',4'-dihydroxy-5,6,7,8-tetramethylflavone > 5-demethylnobiletin > 3'-demethylnobiletin > 4'-demethylnobiletin > nobiletin. It seems that the solubility of PMFs is lower than that of HPMFs. According to the criterion of the lyophilization solubility assay, the solubility of PMFs and HPMFs is low.

Table 3. Solubility of PMFs¹⁰⁴

| Name | LYSA (µg/mL) |
|--|-----------------|
| Tangeretin | 19 |
| Nobiletin | 12 ^a |
| 5,6,7,8,3',4',5'-heptamethoxyflavone | 14 |
| 3,5,6,7,8,3',4'-heptamethoxyflavone | 8 |
| 3'-hydroxy-5,6,7,4'-tetramethoxyflavone | 6 |
| 3'-demthylnobiletin | 29 |
| 4'-demthylnobiletin | 22 |
| 3',4'-dihydroxy-5,6,7,8- tetramethoxyflavone | 53 |
| 3-hydroxy-5,6,7,8,3',4'-hexamethoxyflavone | 37 |
| 5-demthylnobiletin | 32 |

LASA: lyophilization solubility assay

A LYSA number that is greater than 200 µg/mL (maximum reading is 500µg/mL) is defined solubility soluble, and a number between 100µg/mL and 200 lg/mL is defined as medium solubility. Any number that is less than 100µg/mL is defined as low solubility.

^a Data measured using HPLC method.

According to the results of pharmacokinetic studies of nobiletin and tangeretin, they can remain in the blood up to 24 h. When oral gavage 50 mg/kg of nobiletin and tangeretin, nobiletin had a higher serum level (9.3 $\mu\text{g/mL}$) than tangeretin (0.49 $\mu\text{g/mL}$). The author also found that suspended nobiletin had slower absorption and a lower serum level than solubilized nobiletin. Therefore, the solubility of nobiletin would influence the efficacy of oral gavage.¹²³

3.4 Metabolites

Recently, more and more researchers have become interested in the metabolites of phytochemicals. Nielson et al. administrated 100 mg/kg of body weight/day of tangeretin to the rats and then collected their urine and feces. They found 10 different tangeretin metabolites, which are demethylated derivatives of the parent compound, and metabolic changes primarily occurred in the 4' position of the B-ring.^{104, 124} 4'-hydroxy-5,6,7,8-tetramethoxyflavone (4'-OH-TMF) is the primary metabolite from tangeretin, and it may have anti-cancer effects by inhibiting the mTOR pathway.^{104, 125} 5,3'-didemethylnobiletin, 5,4'-didemethylnobiletin, and 5,3',4'-tridemethylnobiletin are three novel urinary metabolites of 5-demethylnobiletin. 5,3'-didemethylnobiletin has a stronger inhibitory effect on human colon cancer cells than its 5-demethylnobiletin counterpart.¹²⁶ 3'-demethylnobiletin, 4'-demethylnobiletin, and 3',4'-didemethylnobiletin

are also metabolites of nobiletin, and they exhibit much stronger anti-inflammatory activity than their parent compound.^{104, 113}

3.5 Prodrug concept and acetylated tangeretin

Prodrugs are derivatives of drug molecules and release their active parent drug *in vivo* to perform their pharmaceutical effects by enzymatic and/or chemical transformation.¹²⁷

Prodrugs provide another solution for potential drugs to conquer different barriers to drug formulation and delivery like poor aqueous solubility, chemical instability, insufficient oral absorption, rapid pre-systemic metabolism, inadequate brain penetration, toxicity and local irritation.¹²⁷ Chemical modification is one of method to improve the physicochemical, biopharmaceutical or pharmacokinetic properties of pharmacologically potent compounds and release active parent drugs by transformation *in vivo* to increase the developability and usefulness of a potential drug¹²⁷⁻¹²⁹. Salicylates are one of the classical examples of natural product-derived remedies to become modern pharmacological agents to reduce the fever, pain and inflammation of rheumatic fever.¹³ However, high doses of sodium salicylate and salicylic acid would cause gastric irritation, therefore the pharmaceutical industry want to find a derivatives that have better efficacy and more tolerable. In 1895, Felix Hoffman, he got acetylsalicylic acid as we know Aspirin by acetylating the hydroxyl group on the benzene ring of salicylic acid and

Aspirin does has better efficacy than salicylic acid confirmed by clinical trials.¹³⁰ Recent research has shown that some acetyl derivatives of compounds exhibit more efficacious biological activity than the original skeletons, such as epigallocatechin-3-gallate (EGCG) and resveratrol.¹³¹⁻¹³³ Wang et al. used chemical modification to obtain 5-acetyl-6,7,8,4'-tetramethylnortangeretin, and it showed a better anti-breast cancer ability than tangeretin.¹³⁴

CHAPTER 4. HYPOTHESIS AND EXPERIMENTAL DESIGN

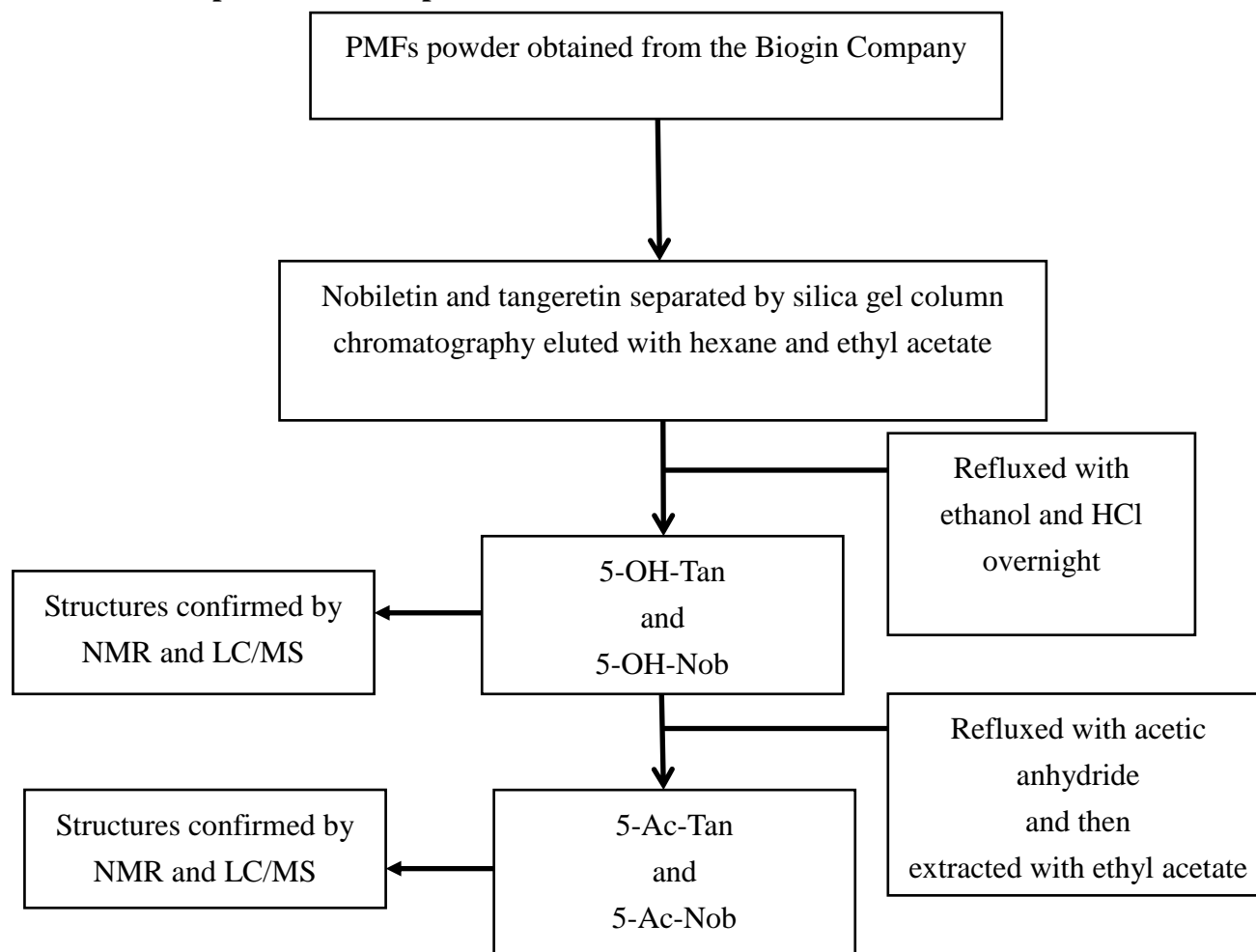
4.1 Hypothesis

Obesity is a serious health problem around the world. Controlling body weight and decreasing lipid accumulation in the body could help to decrease the prevalence of obesity. Diet modification and physical activity are easy methods that we can apply to control our body weight. If we could find some compounds from natural foods that could alleviate lipid accumulation in the body, it would be another method to control obesity. The literature shows that PMFs and HPMFs have anti-cancer, anti-inflammatory, and anti-atherogenic effects and regulate lipid metabolism. Research also indicates that HPMFs and some of HPMFs are metaboite have better biological activities than their parent compounds. However, their structures give them poor aqueous solubility and low bioavailability. Based on the prodrug concept and acetylated tangeretin also has better

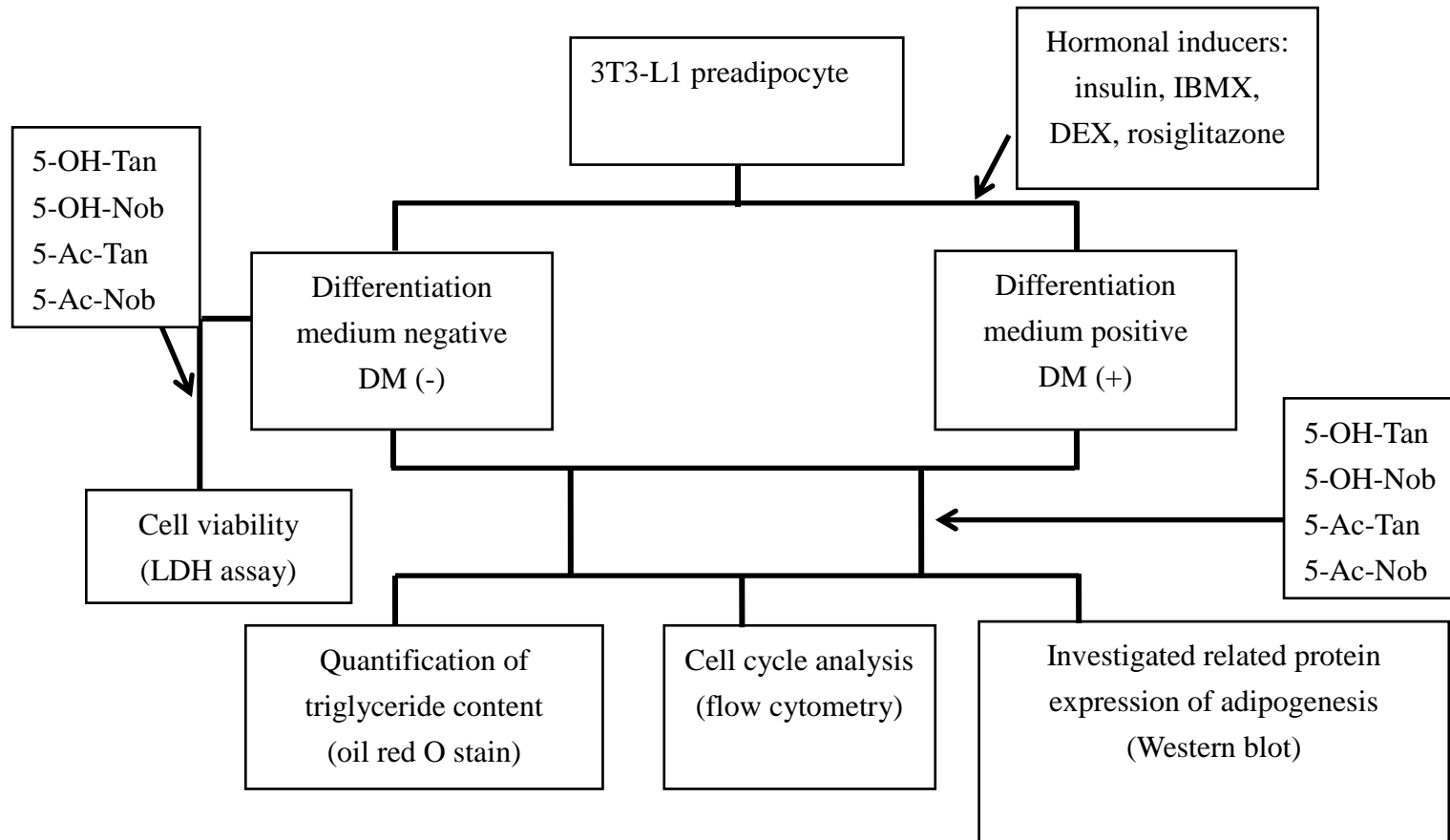
anticancer activity than tangeretin. Therefore, acetylation could be a solution for the low bioavailability of PMFs and HPMFs to make them have better efficacy in the body. In the current study, we used chemical modification to obtain 5-demethyltangeretin (5-OH-Tan), 5-demethylnobiletin (5-OH-Nob), 5-acetyloxy-6,7,8,4'-tetramethoxyflavone (5-Ac-Tan), and 5-acetyloxy-6,7,8,3',4'-pentamethoxyflavone (5-Ac-Nob) from nobiletin and tangeretin, individually. We assumed that the 5-hydroxylated and 5-acetylated forms of nobiletin and tangeretin both could decrease lipid accumulation but that the 5-acetylated form would be more efficacious than the 5-hydroxylated form.

4.2 Experimental design

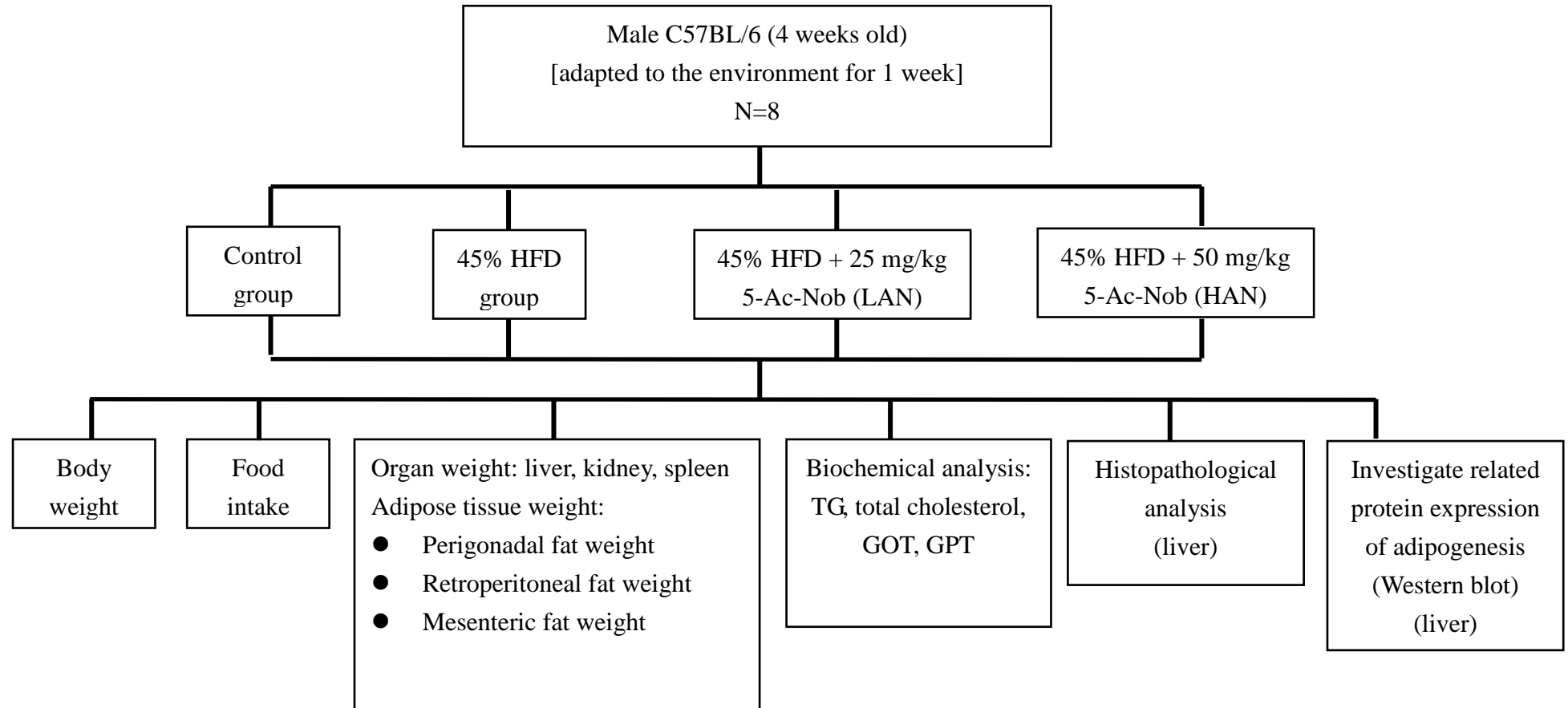
4.2.1 Preparation of compounds



4.2.2 Anti-adipogenesis activity in 3T3-L1 preadipocyte



4.2.3 Anti-adipogenesis activity in an HFD-induced obesity animal model



CHAPTER 5. MATERIALS AND METHOD

5.1 Solvents, reagents, and supplies

PMF powders were obtained from the BioGin Company (BioGin Biochemicals Co., Ltd, Sichuan, China). Methanol, ethanol, water, acetonitrile, chloroform, ethyl acetate, hexanes, 2-propanol, diethyl ether, and acetone of HPLC grade were purchased from Fisher Scientific (Springfield, NJ). Thin layer chromatography (TLC) plates were purchased from Analtech, (Newark, DE, USA). An enhanced chemiluminescent-based detection system (ECL) and hybond-polyvinylidene difluoride (PVDF) membrane were purchased from Millipore (Billerica, MA). Acrylamide, pre-stained protein markers, dimethyl sulfoxide (DMSO), glycerol, and tris-HCl were purchased from Merck (Whitehouse Station, NJ). Bio-Rad protein assay reagent was purchased from Bio-Rad Lab (Hercules, CA). Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, fetal calf serum (FCS), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Anti- β -actin antibody, insulin, 3-isobutylmethylxanthine (IBMX), and dexamethasone (DEX), isopropanol were purchased from the Sigma Chemical Co. (St. Louis, MO). Anti-phospho AMPK, anti-AMPK, and anti-FAS antibodies were purchased from Cell Signaling Technology (Beverly, MA). Other antibodies used in this study were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA).

5.2 PMFs preparation

The PMFs' crude extract was purified by column chromatography packed with silica gel (4:1 hexane:ethyl acetate) to obtain tangeretin and nobiletin as white powders. 5-demethyltangeretin (5-OH-Tan) or 5-demethylnobiletin (5-OH-Nob) was prepared by refluxing tangeretin or nobiletin with 6 N HCl in 95% ethanol overnight. The 5-acetyloxy-6,7,8,4'-tetramethoxyflavone (5-Ac-Tan) and 5-acetyloxy-6,7,8,3',4'-pentamethoxyflavone (5-Ac-Nob) were prepared by refluxing 5-OH-Tan or 5-OH-Nob with acetic anhydride overnight and then extracted with ethyl acetate. NMR spectra were recorded on a Bruker AVIII 500 MHz FT-NMR (Bruker, Rheinstetten, Germany) in CDCl₃. LC/MS was performed by a Thermo Scientific Linear Ion Trap Mass Spectrometer (LXQTM) in positive ionization mode (Thermo Scientific, Waltham, MA, USA). I appreciate the kind help of Dr. Guor-Jien Wei from Kainan University in Taiwan for preparing these compounds.

5.3 Cell culture

Mouse 3T3-L1 preadipocytes purchased from the American Type Culture Collection (Rockville, MD) were grown in DMEM medium supplemented with 2 mM glutamine (GIBCO BRL), 1% penicillin/streptomycin (10,000 units of penicillin/mL and 10 mg

streptomycin/mL), and 10% fetal calf serum (FCS) in a 10 cm dish (Nunc Thermo, Waltham, MA) at 37 °C under a humidified 5% CO₂ atmosphere thermo incubator (Waltham, MA).

5.4 Cell differentiation

For the 3T3-L1 preadipocytes to become mature adipocytes, eight days were needed after the addition of differentiation medium (DM) includes insulin, 3-isobutylmethylxanthine (IBMX), dexamethasone (DEX), and rosiglitazone. To start the differentiation of the 3T3-L1 preadipocytes, cells were seeded into a 96-well (2.5×10^4 /mL) plate or a 10 cm dish and cultured as described above. After two days of being cultured in the DMEM medium with 10% fetal calf serum (FCS), the medium was then changed to a 10% fetal bovine serum (FBS) DMEM medium. After confluence, defined as day 0, cells were incubated in DM containing 5 µg/mL insulin, 0.5 mM IBMX, 1 µM DEX, and 2 µM rosiglitazone in DMEM medium containing 10% fetal bovine serum (FBS) for 48 h. After two days, the cell culture in the DMEM medium contained 10% FBS and 5 µg/mL insulin for another two days, defined as day 2. Starting at day 4, cells were incubated in a DMEM medium containing 10% FBS as a DM positive [DM (+)] group, and the medium was changed every two days until day 8. The control group cells were not cultured with

insulin, IBMX, and DEX at day 0 and insulin at day 2 as a DM negative [DM (-)] group.

Different concentrations of 5-OH-Nob, 5-OH-Tan, 5-Ac-Tan, and 5-Ac-Nob were cultured with cells from day 0 with insulin, IBMX, and DEX and co-cultured with insulin at day 2, and then the medium was changed with these compounds in different concentrations every two days until day 8.

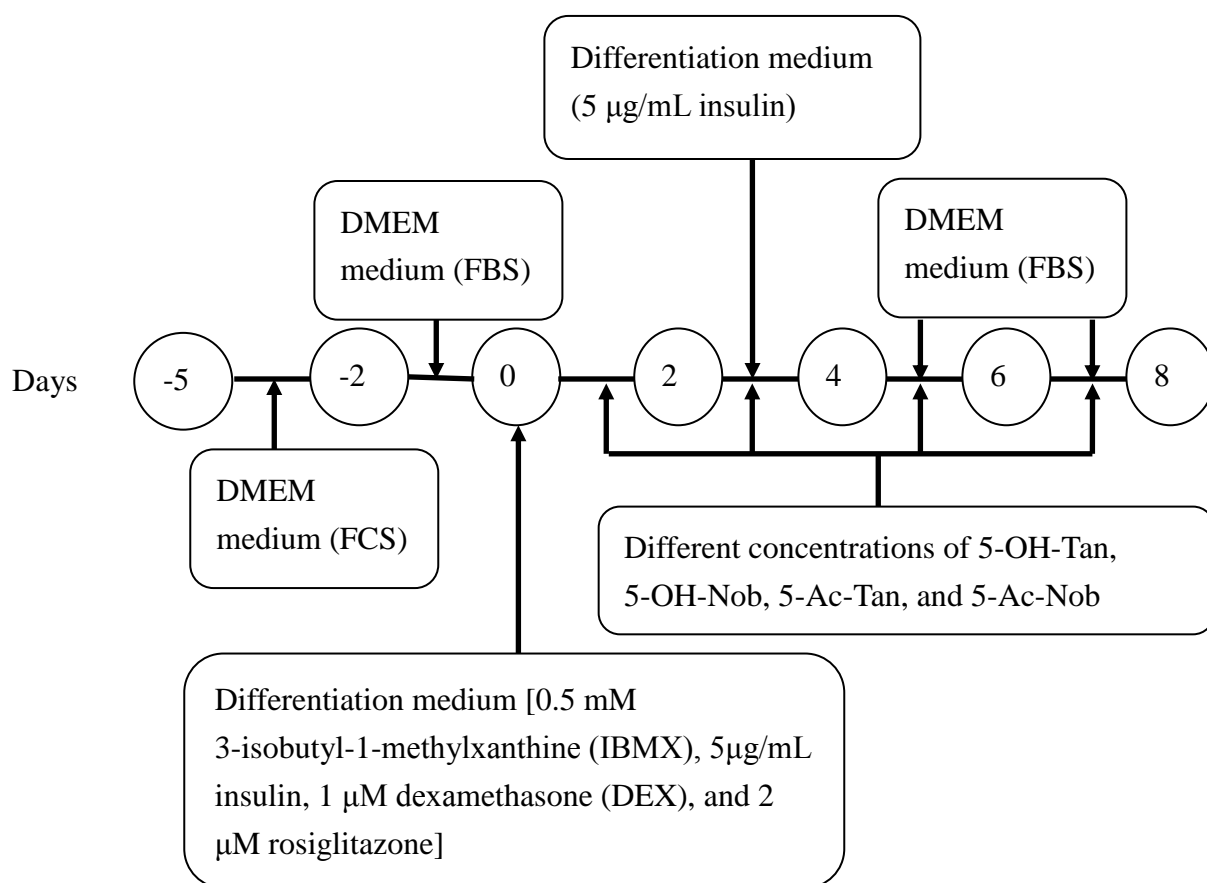


Figure 7. The experimental process of 3T3-L1 preadipocyte cell differentiation

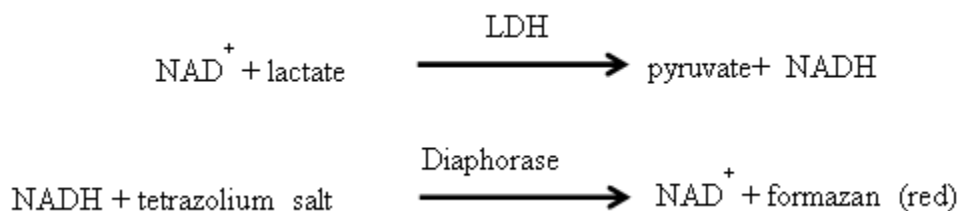
5.5 Cell viability

Cell viability was determined by measuring the lactate dehydrogenase (LDH) released.

LDH is a cytoplasmic enzyme released in the cellular supernatant when cells die, and its

activity is in proportion with cell death.¹⁴¹ We used the CytoTox 96® Non-Radioactive Cytotoxicity Assay purchased from the Promega Company (San Luis Obispo, CA, USA).

The chemical reactions of the CytoTox 96® Assay are as follows:



3T3-L1 preadipocytes were incubated in the DMEM with 10% FCS with and without different concentrations of 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob. After 48 hrs of incubation, we followed the instructions for the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit: transfer 50 µL supernatant to a 96-well plate, add 50 µL reconstituted Substrate Mix to each well, cover plate, and incubate 30 minutes at room temperature; add 50 µL Stop Solution to each well and then quantify at absorbance 490 nm. Determine the percentage of cell death using the following formula:

$$100\% - \% \text{Cytotoxicity} = \text{Experimental LDH release} / \text{Maximum LDH release}.$$

5.6 Cell cycle analysis

The cell cycle has G₁, S, G₂, and M phases. In the G₁ phase, the cell prepares for DNA synthesis. In the S phase, the cell synthesizes DNA; therefore, it has aneuploidy DNA between 2N and 4N. In the G₂ phase, the cell prepares for mitosis. In the M phase, the

cell undergoes mitosis. G_0 cells are not in the cell cycle; they are among the non-growing, non-proliferating cells.

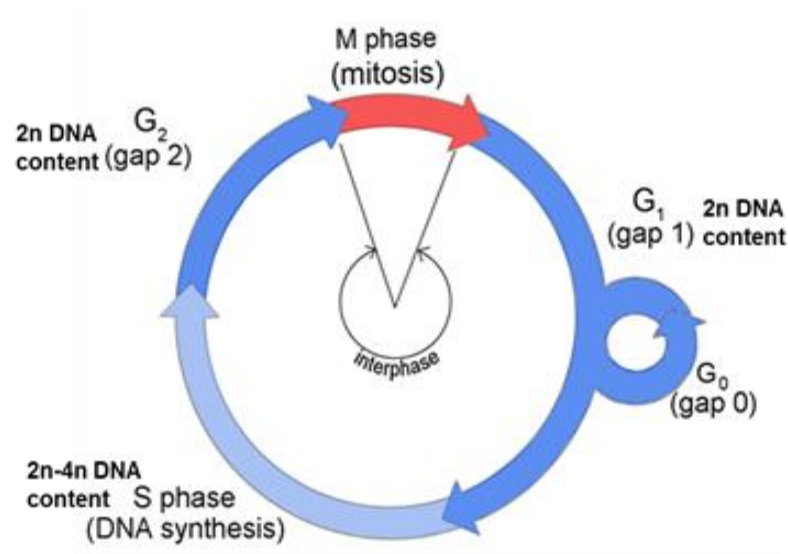


Figure 8. The cell cycle¹³⁵

Analysis of the cell cycle by flow or cytometric methods has been developed for several years. The method is based on measuring cellular DNA content. Propidium iodide (PI) is the DNA fluorochrome; it requires blue light as the excitation source (e.g., 488 nm argon ion laser). The results show the percentage of cells in the G₁, S, and G₂/M phases. Apoptotic cells have fractional DNA content because they have fragmented DNA, and it is presented in the percentage as “sub-G₁” in the figure. It’s unclear what figure is referred to.¹³⁶⁻¹³⁸

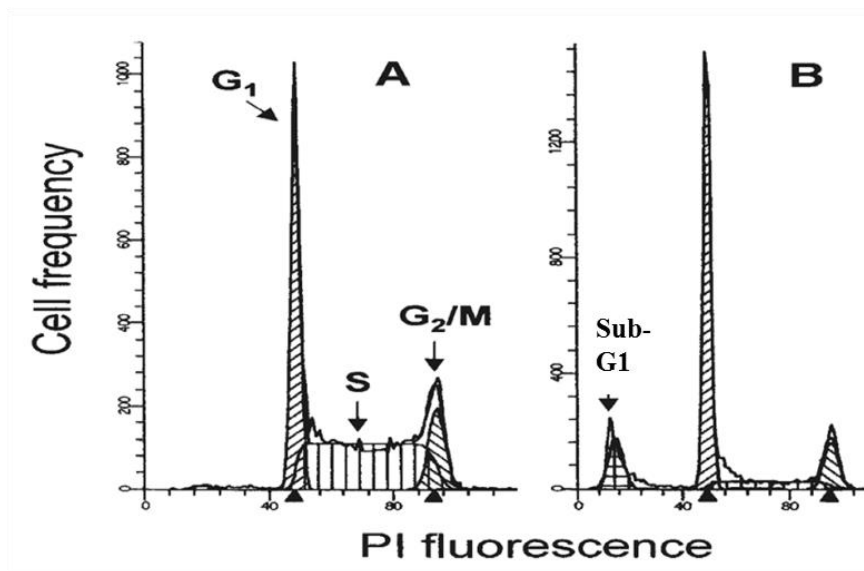


Figure 9. Detection of cell cycle by flow cytometry¹³⁷

3T3-L1 cells were cultured in 10 cm dishes and incubated with or without differentiation medium, 5-OH-Tan, 5-OH-Nob, 5-Ac-TMF, and 5-Ac-PMF for 24 h. The cells were washed with 200 μ L PBS and harvested by 200 μ L trypsin and suspended in PBS. They were fixed with 800 μ L 100% iced ethanol stored at 20 °C. Before analysis by Beckman Coulter FC500 flow cytometry (Indianapolis, IN), the cells were washed with PBS and centrifuged at 1,500 rpm for 5 minutes. The cells were then suspended in 500 μ L PI solution (0.2 mg/mL propidium iodide, 0.5% Triton X-100 in PBS, and 0.5 mg/mL RNase) and incubated at 37 °C for 30 min.

5.7 Oil red O staining

Intracytoplasmic lipid accumulation is proportional to the status of differentiation. Oil red

O stain is a common method to quantify lipid content in adipose. Oil red O is a dye that is soluble in lipids and a histochemical stain for neutral fats and cholesteryl esters.^{63, 139} The 3T3-L1 cells stained with oil red O at day 8 to quantify the triacylglycerol content in the cells. Cells were washed with phosphate-buffered saline (PBS) twice and then fixed with 10% formalin overnight at 4 °C. The oil red O stock solution, dissolved in isopropanol (5 mg/mL), was filtered through a 0.2 µm filter. Fresh oil red O solution was diluted with distilled water at a ratio of 3 to 2. Cells were stained with oil red O for 5 minutes at room temperature and then washed three times with PBS. Lipids extracted from the cells by isopropanol were quantified at 510 nm absorbance.

5.8 Animal experiments

Male C57BL/6J mice were purchased from the BioLASCO Experimental Animal Center (Taiwan Co., Ltd., Taipei, Taiwan) at four weeks of age. The mice were housed in a controlled temperature (24 ± 2 °C) and 55% relative humidity with a 12 h light/dark cycle, and the mice had free access to water and food. After one week of acclimation, the animals were randomly divided into four groups of eight animals each as follows: control diet (control, 15% of calories from fat), high-fat diet (HFD; 45% of calories from fat: 19.6 g lard, 2 g soybean oil, and 0.5% cholesterol per 100 gram in the normal diet), oral gavage 25 mg/kg 5-Ac-Nob with HFD as a LAN group, and oral gavage 50 mg/kg

5-Ac-Nob with HFD as a HAN group for 13 weeks. The experimental diets were modified from the LabDiet 5001 standard diet (LabDiet, St. Louis, MO, USA); the nutritional composition of the food is listed in Table 4. The food intake was measured every day, and the body weight was recorded every week for 13 weeks. At the end of the study, all the animals were fasted overnight and then sacrificed by CO₂ asphyxiation. Blood samples were collected from the heart for biochemical analysis. Liver, spleen, kidney, and abdominal fat pads (perigonadal, retroperitoneal, and mesenteric fat) were collected and weighed.

Table 4. Composition of Diets

| Macronutrient Composition | Control | HFD |
|------------------------------|---------|------|
| Carbohydrate % of energy | 58.3 | 37.5 |
| Protein % of energy | 27.2 | 17.5 |
| Fat % of energy | 14.6 | 45 |

5.9 Biochemical analysis

Plasma samples were separated by centrifugation at 3,000 rpm for 10 min at 4 °C and stored at -80 °C until analysis. Plasma GOT, GPT, TG, and total cholesterol were measured by a commercial assay kit. Plasma samples were spotted onto separate Fujifilm Dri-Chem slides (Fujifilm, Kanagawa, Japan), and each biochemical indicator was

determined by a Fujifilm Dri-Chem 3500s blood biochemistry analyzer (Fujifilm, Kanagawa, Japan) according to the manufacturer's protocol.

5.10 Measurement of liver TG level

Liver TG levels were measured using a commercial kit (Cayman Chemical, Ann Arbor, MI, USA). 50 mg of liver were homogenized with diluted standard diluent containing protease inhibitor, and then the manufacturer's protocol was followed.

5.11 Histopathological examinations

A portion of the median lobe of the liver was dissected and fixed in 10% buffered formalin for at least 24 h, dehydrated with a sequence of ethanol solutions, and processed for embedding in paraffin. Paraffin sections were cut to 5 μ m in thickness and stained with hematoxylin and eosin (H&E), and the paraffin sections were observed under photo microscopy. All the histopathological examinations were performed by Professor Jiunn-Wang Liao (National Chung Hsing University, Taichung, Taiwan).

5.12 Western blotting

3T3-L1 cells were incubated in a 10 cm dish and harvested at day 8. Liver tissue and cell proteins were extracted by adding gold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10

µg/mL leupeptin) on ice for 1 hr and then centrifuging at 12,000 rpm for 30 minutes at 4 °C. The protein was quantified by Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany). 25 µg of protein were mixed with 5× sample dye and boiled at 100 °C for 10 min and then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After 3 h, the SDS-PAGE was transferred to the PVDF membranes (Millipore Corp., Bedford, MA) with transfer buffer containing 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were put in blocking solution containing 20 mM Tris-HCl buffer with 1% bovine serum albumin at room temperature for 1 h and then applied primary antibodies including LKB1, phosphorylated-LKB1, AMPK, phosphorylated-AMPK, SREBP1, phosphorylated-ACC, fatty acid synthesis (FAS), and β-actin (Cell Signaling Technology, Beverly, MA). The membranes were washed three times with PBST buffer for 10 min each, and then the membranes were incubated with a 1:5,000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA) and washed again with PBST buffer three times. The transferred proteins were visualized using an enhanced chemiluminescence detection kit (ECL) (Millipore Corp., Bedford, MA).

5.13 Analysis of nobiletin, 5-demethyl and

5-acetyloxy-6,7,8,3',4'-pentamethoxyflavone in mouse plasma by LC-MS

Male ICR mice were purchased from the BioLASCO Experimental Animal Center (Taiwan Co., Ltd., Taipei, Taiwan) at 6 weeks of age. The mice were housed in a controlled temperature (24 ± 2 °C) and 55% relative humidity with a 12 h light/dark cycle and the mice free access to water and food. After 1 week of acclimation, mice were administered with 100 mg/ kg bw of 5-OH-Nob or 5-Ac-Nob individually by oral gavage. Mice were sacrificed and the blood was collected by cardiac puncture after 15, 30, 45 and 60 min administration and then stored at -80 °C until analysis. The plasma was collected by centrifugation at 3000 rpm for 10 minutes. Two hundred uL plasma was extracted with 1 mL ethyl acetate (EA). The supernatant layer was collected after centrifugation at 14000 x g for 10 min and the solvent was removed by a stream of nitrogen gas. The residue was reconstituted in 200 μ L methanol and followed by LC/MS. LC/MS was performed by a Thermo Scientific Linear Ion Trap Mass Spectrometer (LXQTM) in positive ionization mode (Thermo Scientific, Waltham, MA, USA).

5.14 Statistical analysis

The data were presented as the mean \pm SD and analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple comparison test (SAS Institute Inc., Cary, NC) to

determine the significant difference among groups ($P < 0.05$).

CHAPTER 6. RESULTS

6.1 Structure of 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob

The structures of 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob are shown in Figure 22.

^1H -NMR spectrum of 5-OH-Tan: $\delta = 3.87$ (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 4.09 (3H, s, OCH_3), 6.57 (1H, s, H-3), 7.00 (2H, d, $J = 8.4$ Hz, H-3', and H-5'), 7.59 (2H, d, $J = 8.4$ Hz, H-2', and H-6'), and 12.55 (1H, s, OH) (Figure 10). ^1H -NMR spectrum of 5-OH-Nob: $\delta = 3.93$ (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 3.95 (6H, s, OCH_3), 4.09 (3H, s, OCH_3), 6.58 (1H, s, H-3), 6.97 (1H, d, $J = 8.5$ Hz, H-2'), 7.39 (1H, d, $J = 2.1$ Hz, H-5'), 7.55 (1H, dd, $J = 2.1$, and 8.5 Hz, H-6'), and 12.51 (1H, s, OH) (Figure 14). ^1H -NMR spectrum of 5-Ac-Tan: $\delta = 2.48$ (3H, s, $(\text{CO})\text{CH}_3$), 3.88 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 4.05 (3H, s, OCH_3), 4.10 (3H, s, OCH_3), 6.54 (1H, s, H-3), 7.02 (2H, d, $J = 9.0$ Hz, H-3', and H-5'), 7.85 (2H, d, $J = 9.0$ Hz, H-2' and H-6') (Figure 12). ^1H -NMR spectrum of 5-Ac-Nob: $\delta = 2.45$ (3H, s, $(\text{CO})\text{CH}_3$), 3.86 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 4.03 (3H, s, OCH_3), 4.08 (3H, s, OCH_3), 6.52 (1H, s, H-3), 6.96 (1H, d, $J = 8.5$ Hz, H-2'), 7.36 (1H, d, $J = 2.1$ Hz, H-5'), and 7.52 (1H, dd, $J = 2.1$,

8.5 Hz, H-6') (Figure 16). Mass spectrometry showed that 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob exhibited protonated molecular ions $[M+H]^+$ at m/z 359.24, 389.28, 401.07, and 431.08, respectively (Figures 18-21).

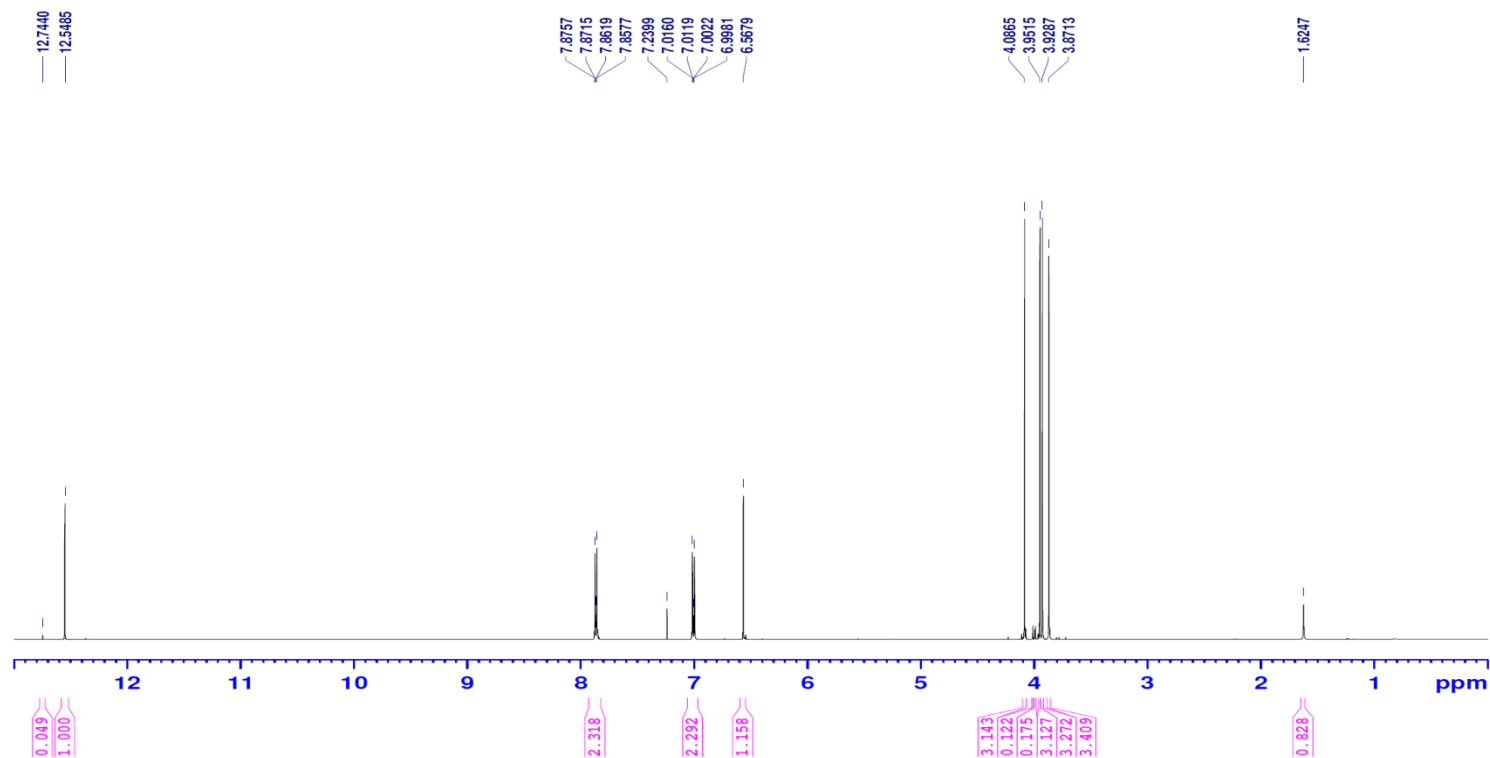


Figure 10. ¹H NMR of 5-OH-Tan

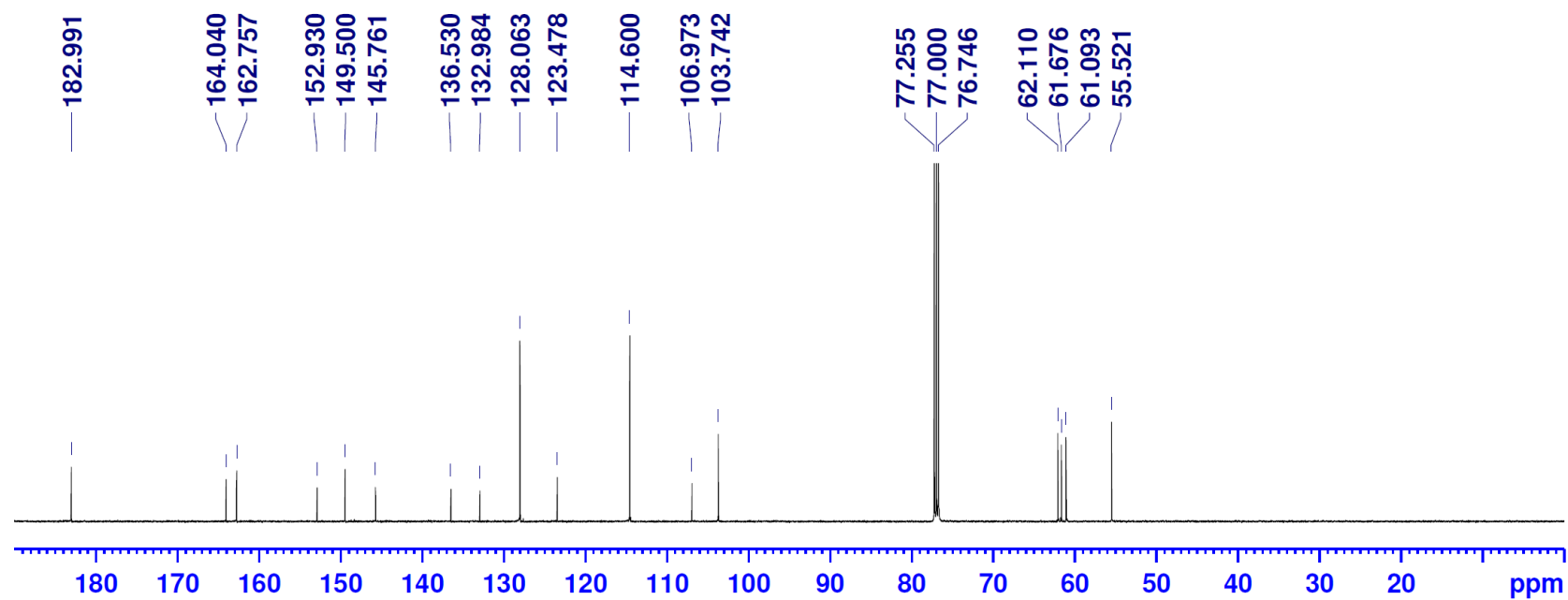


Figure 11. ^{13}C NMR of 5-OH-Tan

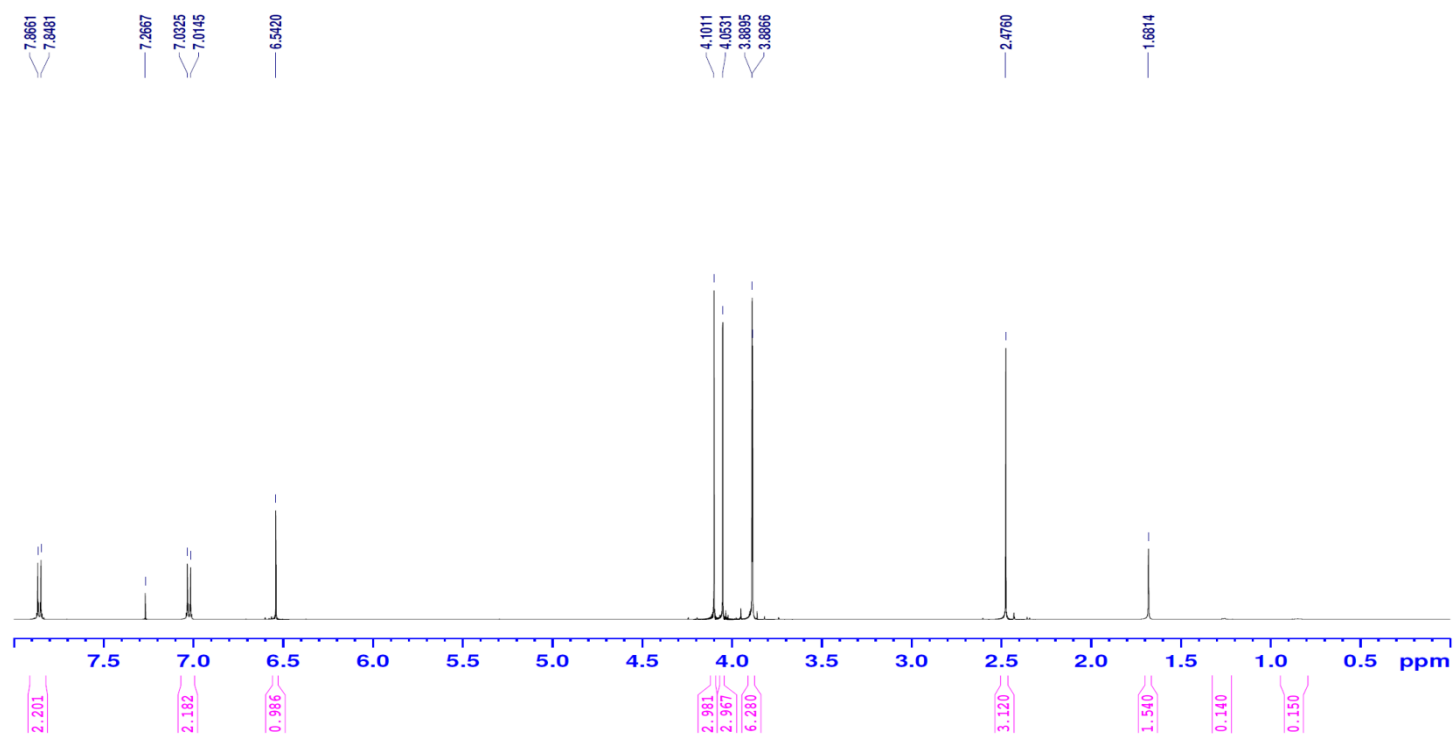


Figure 12. ^1H NMR of 5-Ac-Tan

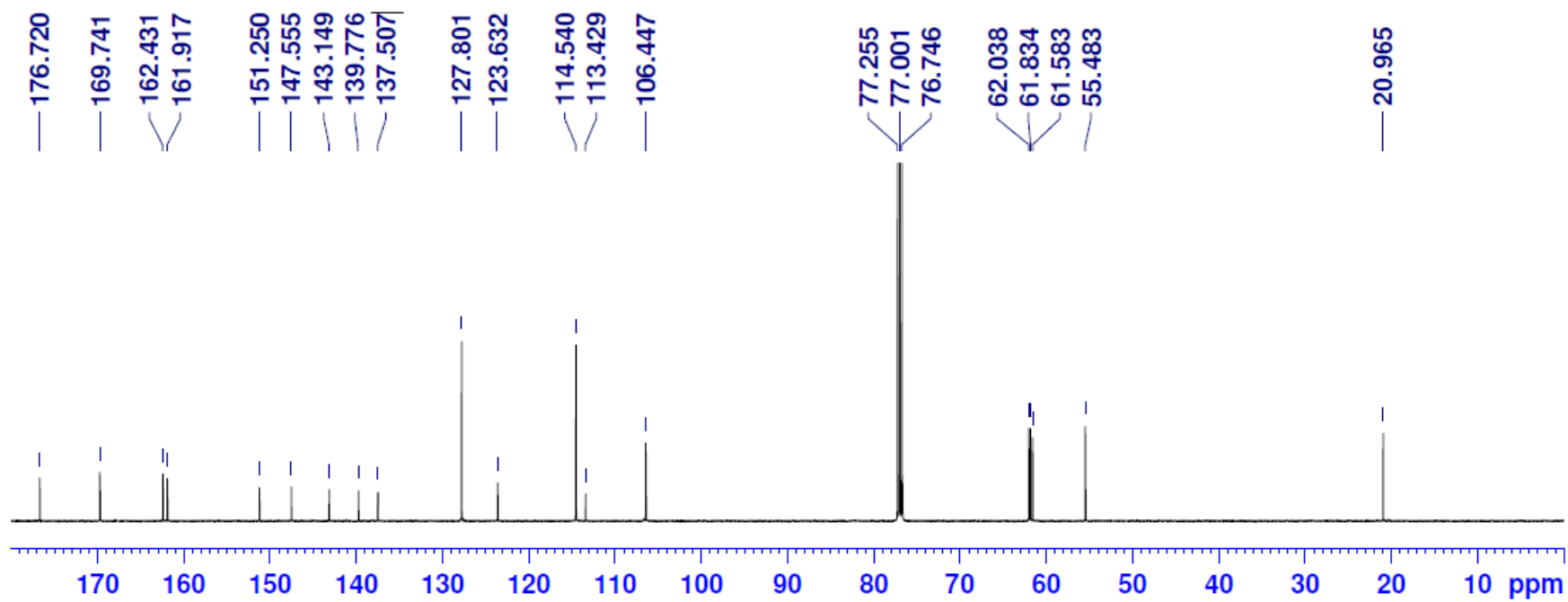


Figure 13. ^{13}C NMR of 5-Ac-Tan

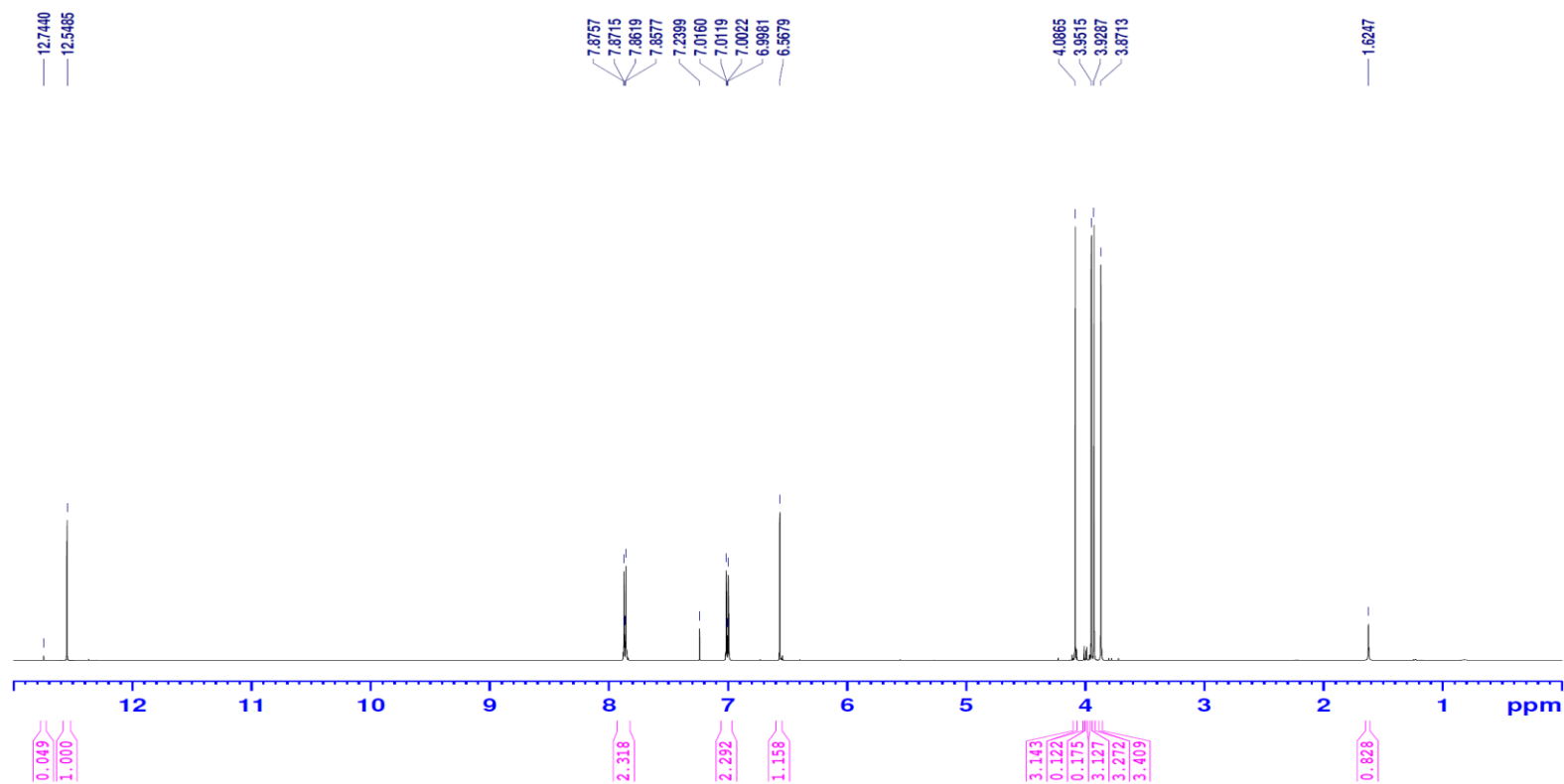


Figure 14. ¹H NMR of 5-OH-Nob

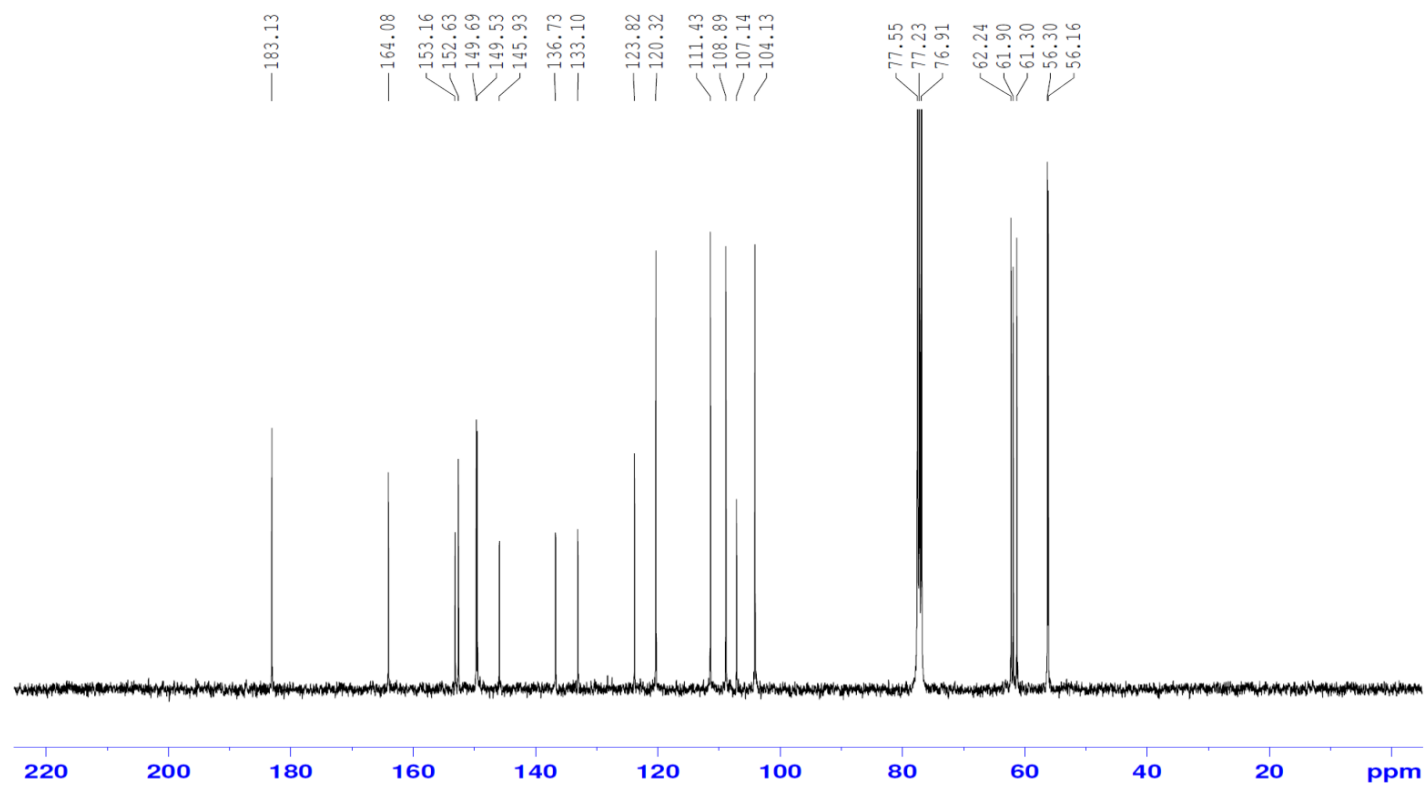


Figure 15. ^{13}C NMR of 5-OH-Nob

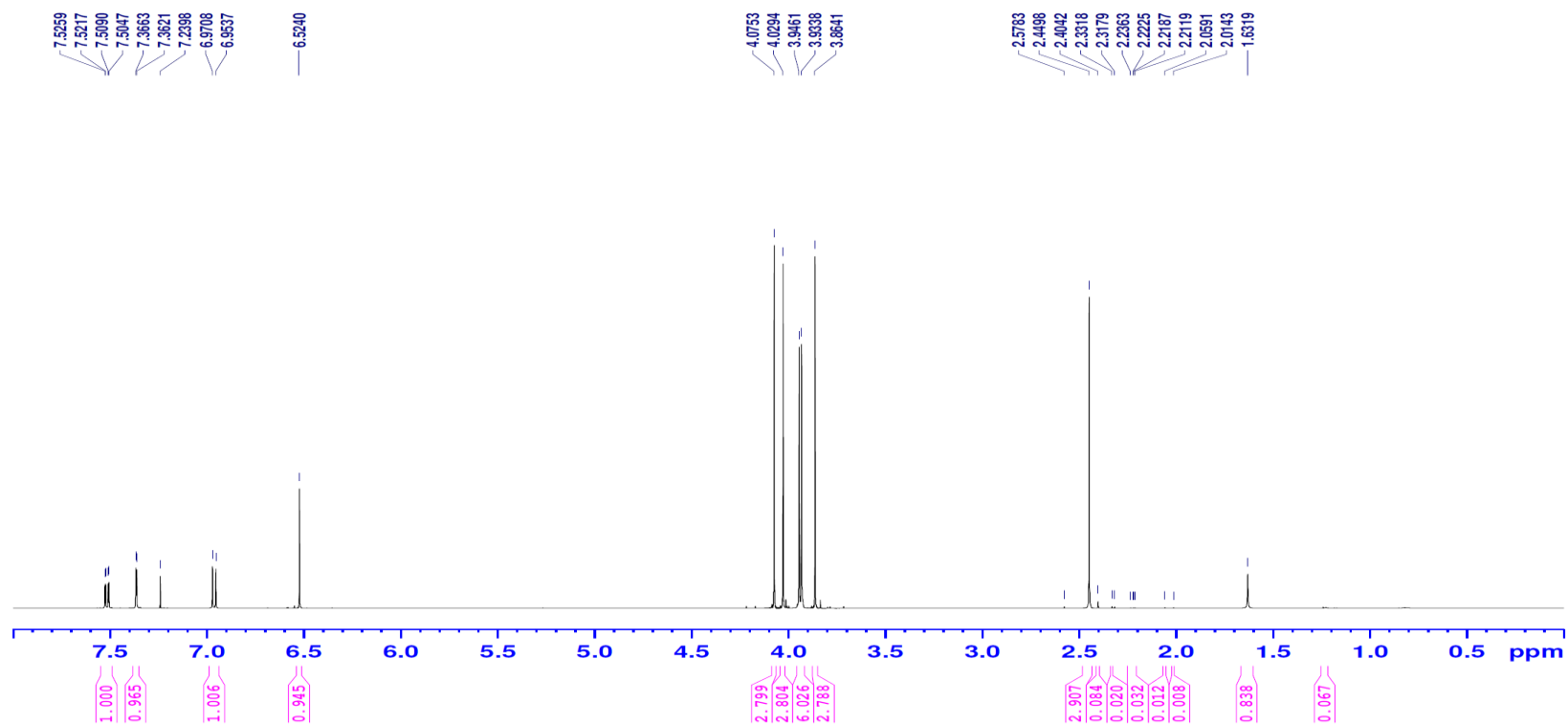


Figure 16. ¹H NMR of 5-Ac-Nob

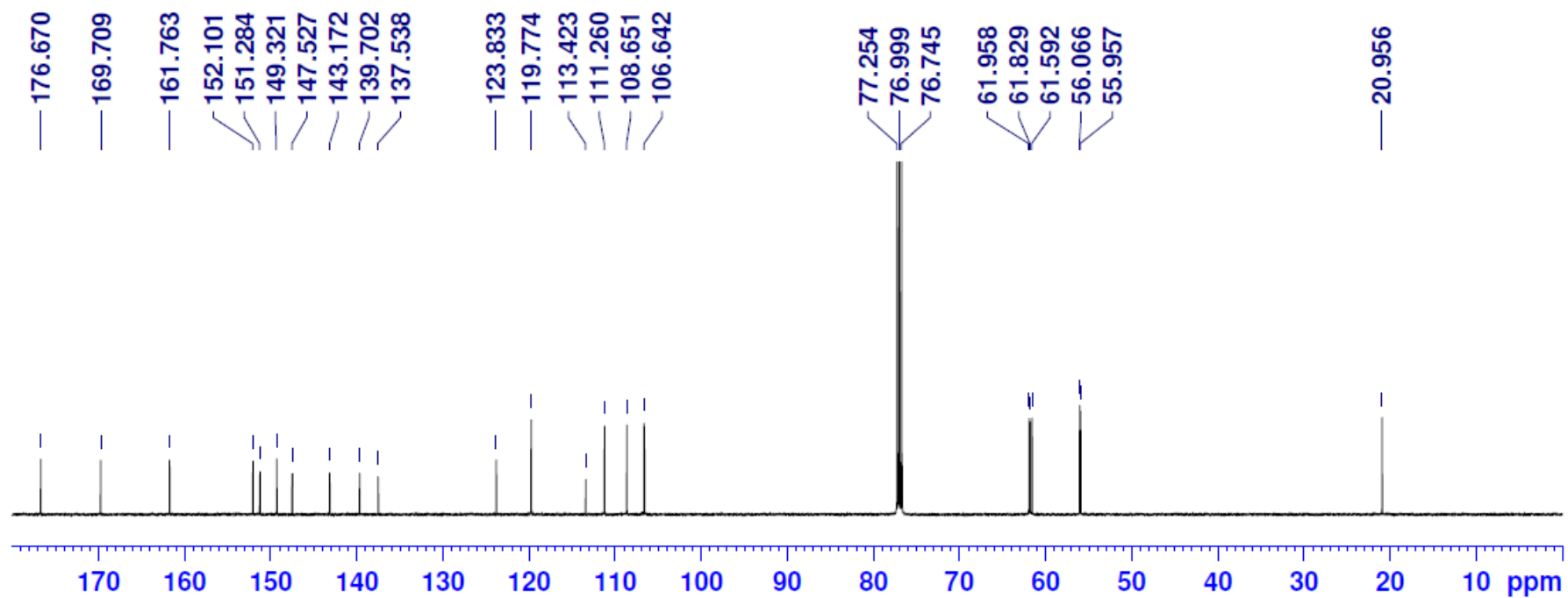


Figure 17. ^{13}C NMR of 5-Ac-Nob

Table 5. ^{13}C NMR of 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob (Figures 8, 10, 12, 14)

| Compounds | C | | | | | | | | | | | | | | | OMe | | | | | (C=O)CH ₃ |
|-----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|------|------|------|------|----------------------|
| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 1' | 2' | 3' | 4' | 5' | 6' | 6 | 7 | 8 | 3' | 4' | 5 |
| 5-OH-Tan | 162.7 | 103.7 | 183.0 | 149.5 | 136.6 | 152.9 | 133.0 | 145.8 | 107.0 | 123.5 | 128.1 | 114.6 | 164.0 | 114.6 | 123.5 | 62.1 | 61.7 | 61.1 | | 55.5 | |
| 5-OH-Nob | 164.1 | 104.1 | 183.1 | 152.6 | 136.7 | 153.2 | 133.1 | 145.9 | 107.1 | 123.8 | 111.4 | 149.5 | 149.7 | 108.9 | 120.3 | 62.2 | 61.3 | 61.9 | 56.3 | 56.2 | |
| 5-Ac-Tan | 161.9 | 106.4 | 176.7 | 147.6 | 139.8 | 151.2 | 137.5 | 143.1 | 113.4 | 123.6 | 127.8 | 114.5 | 162.4 | 114.5 | 127.8 | 62.0 | 61.8 | 61.6 | | 55.5 | 169.7/21.0 |
| 5-Ac-Nob | 161.8 | 106.6 | 176.7 | 147.5 | 139.7 | 152.1 | 137.5 | 143.1 | 113.4 | 123.8 | 111.3 | 149.3 | 151.3 | 108.9 | 120.3 | 62.0 | 61.6 | 61.8 | 56.1 | 56.0 | 169.7/21.0 |

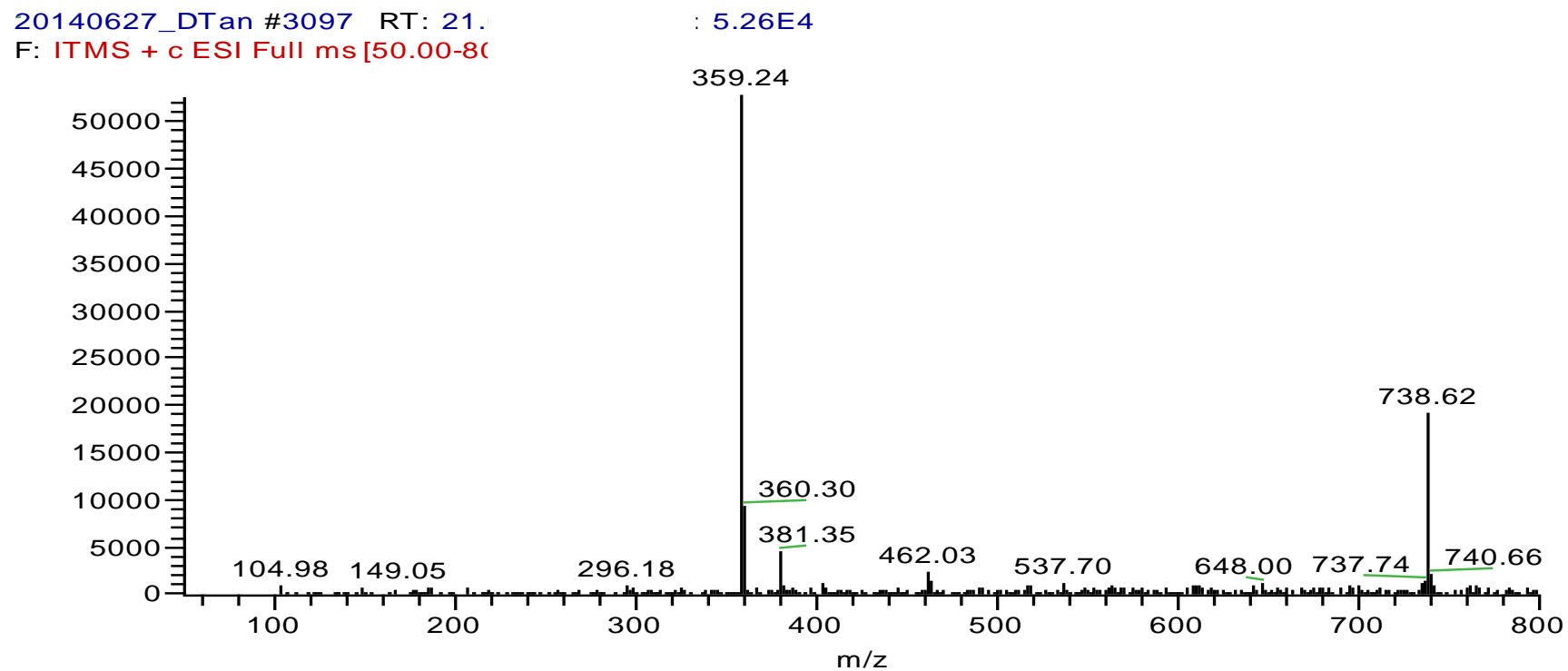


Figure 18. LC-MS of 5-OH-Tan

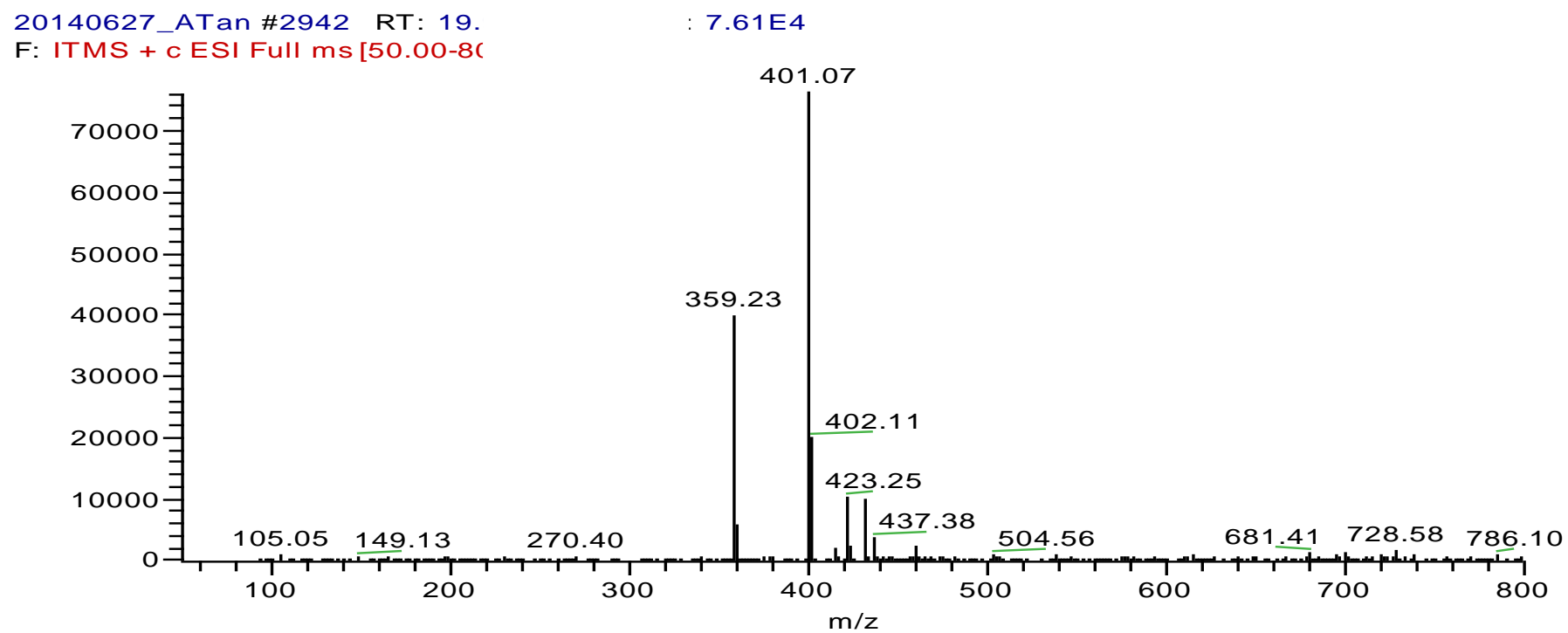


Figure 19. LC-MS of 5-Ac-Tan

20140627_DNob #2964 RT: 20.
F: ITMS + c ESI Full ms [50.00-800]

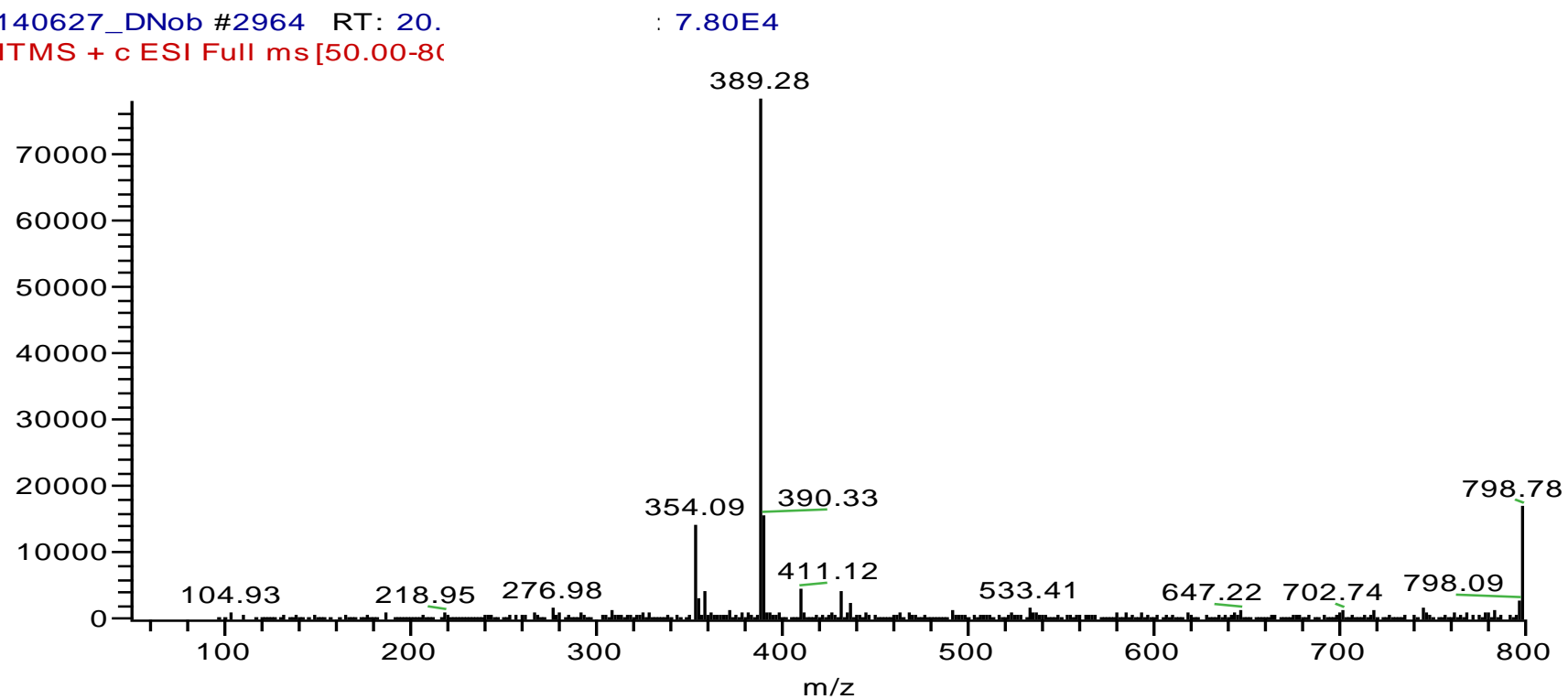


Figure 20. LC-MS of 5-OH-Nob

20140627_ANob #2780 RT: 18.
F: ITMS + c ESI Full ms [50.00-800]

: 1.08E5

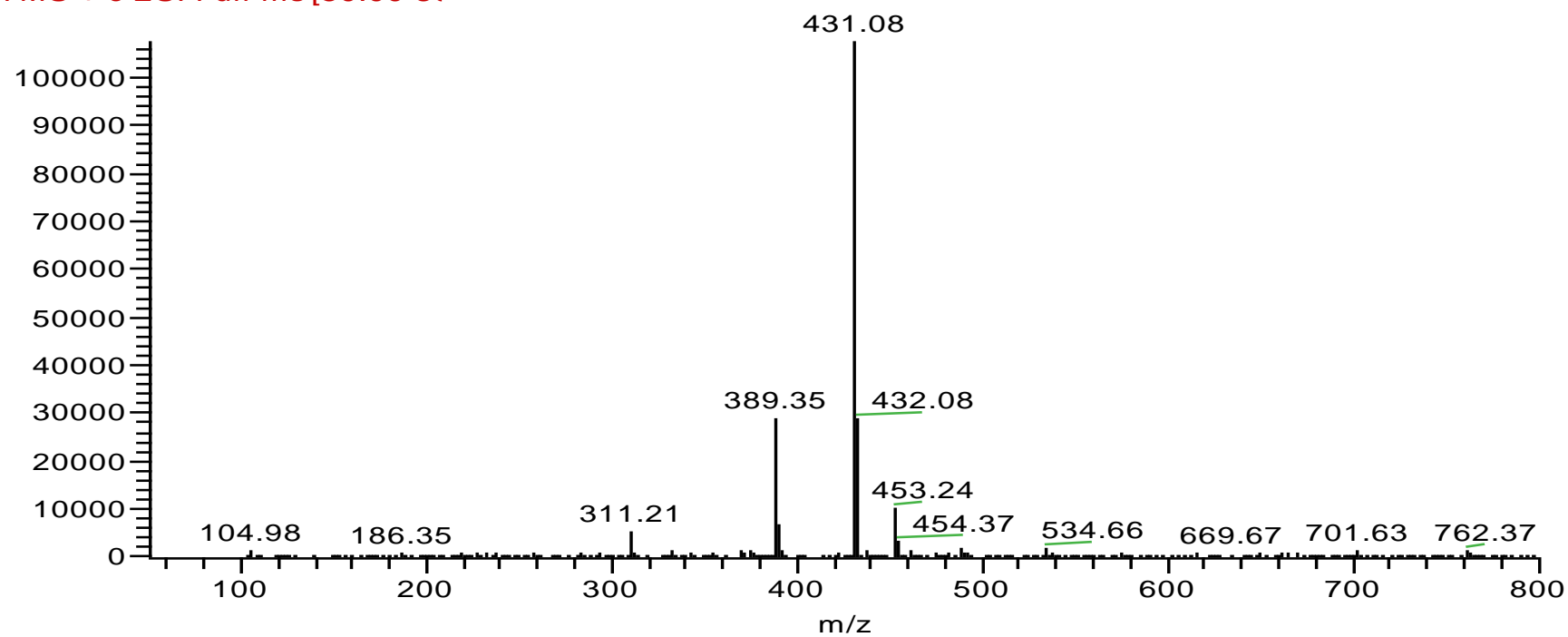


Figure 21. LC-MS of 5-Ac-Nob

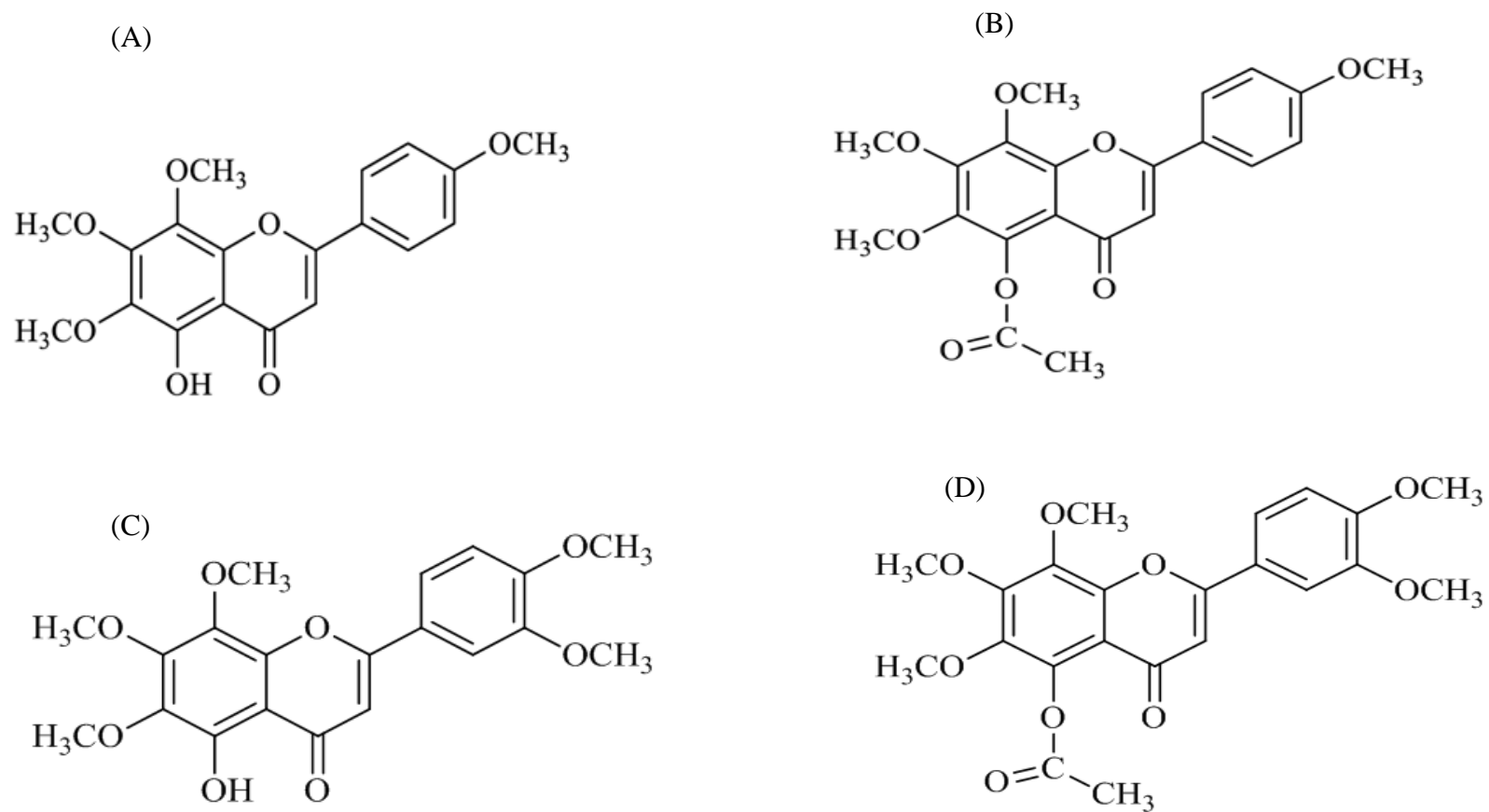


Figure 22. Chemical structure of (A) 5-OH-Tan, (B) 5-Ac-Tan, (C) 5-OH-Nob, and (D) 5-Ac-Nob

6.2 Anti-adipogenesis in a 3T3-L1 preadipocyte model

6.2.1 Cell viability of 3T3-L1

Cell viability was analyzed by LDH. 3T3-L1 cells were treated with 2.5, 5, 10, and 20 μM of 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob for 48 hrs. As shown in Table 7, all these concentrations and compounds showed no significant differences from the control group without adding any compounds to the cells. This means that our compounds did not cause any cytotoxicity in the 3T3-L1 preadipocytes.

Table 6. Cell viability on 3T3-L1

| Concentration | Groups | | | |
|-------------------|--------------------------------|--------------------------------|-------------------------------|-------------------------------|
| | 5-OH-Tan | 5-Ac-Tan | 5-OH-Nob | 5-Ac-Nob |
| Control | 86.41 \pm 3.52 ^a | 86.41 \pm 3.52 ^a | 81.89 \pm 5.69 ^a | 81.89 \pm 5.69 ^a |
| 2.5 μM | 90.43 \pm 2.98 ^a | 87.86 \pm 7.82 ^a | 79.40 \pm 1.60 ^a | 81.30 \pm 5.64 ^a |
| 5 μM | 86.56 \pm 12.96 ^a | 84.87 \pm 5.20 ^a | 81.52 \pm 4.54 ^a | 83.51 \pm 4.37 ^a |
| 10 μM | 84.18 \pm 2.03 ^a | 84.59 \pm 14.89 ^a | 78.88 \pm 7.75 ^a | 78.61 \pm 7.08 ^a |
| 20 μM | 84.62 \pm 2.66 ^a | 79.02 \pm 5.94 ^a | 80.30 \pm 4.62 ^a | 82.86 \pm 7.98 ^a |

The viability of different concentrations of 5-demethyltangeretin (5-OH-Tan), 5-acetyloxy-6,7,8,4'-tetramethoxyflavone (5-Ac-Tan), 5-demethylnobiletin (5-OH-Nob), 5-acetyloxy-6,7,8,3',4'-pentamethoxyflavone (5-Ac-Nob) treated with 3T3-L1 cells for 48 h and analyzed by LDH assay. Data are expressed as mean \pm SD (n=3) and analyzed statistically using one-way ANOVA and Duncan's test. Different letters (a-b) represent statistically significant differences among treatments, $p < 0.05$.

6.2.2 Triacylglycerol content in a 3T3-L1 preadipocyte model

After 8 days of differentiation, the preadipocytes became mature adipocytes, and the cells were treated with 2.5, 5, 10, and 20 μ M of 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob over those 8 days. The triacylglycerol content for the DM (-) group was 23%, and the triacylglycerol content for the 2.5, 5, 10, and 20 μ M 5-OH-Tan groups was 86, 86, 79, and 78%, respectively, compared with the DM (+) group (Figure 23). The triacylglycerol content for the DM (-) group was 26%, and the triacylglycerol content for the 2.5, 5, 10, and 20 μ M 5-Ac-Tan groups was 89, 80, 74, and 40%, respectively, compared with the DM (+) group (Figure 24). The triacylglycerol content of the DM (-) group was 24%, and the triacylglycerol content for the 2.5, 5, 10, and 20 μ M 5-OH-Nob groups was 90%, 78, 65, and 59%, respectively, compared with the DM (+) group (Figure 25). The triacylglycerol content of the DM (-) group was 26%, and the triacylglycerol content for the 2.5, 5, 10, and 20 μ M of 5-Ac-Nob groups was 89, 78, 53, and 28%, respectively, compared with the DM (+) group (Figure 26). According to the results of the triacylglycerol content testing, 2.5 and 5 μ M did not obviously decrease the triacylglycerol content in each compound. At 10 μ M, 5-OH-Nob and 5-Ac-Nob decreased triacylglycerol content more than 5-OH-Tan and 5-Ac-Tan, respectively. At 20 μ M, the percentage of triacylglycerol content exhibited the following order: 5-OH-Tan > 5-OH-Nob > 5-Ac-Tan > 5-Ac-Nob. 20 μ M 5-Ac-Nob showed more anti-adipogenesis activity than other compounds.

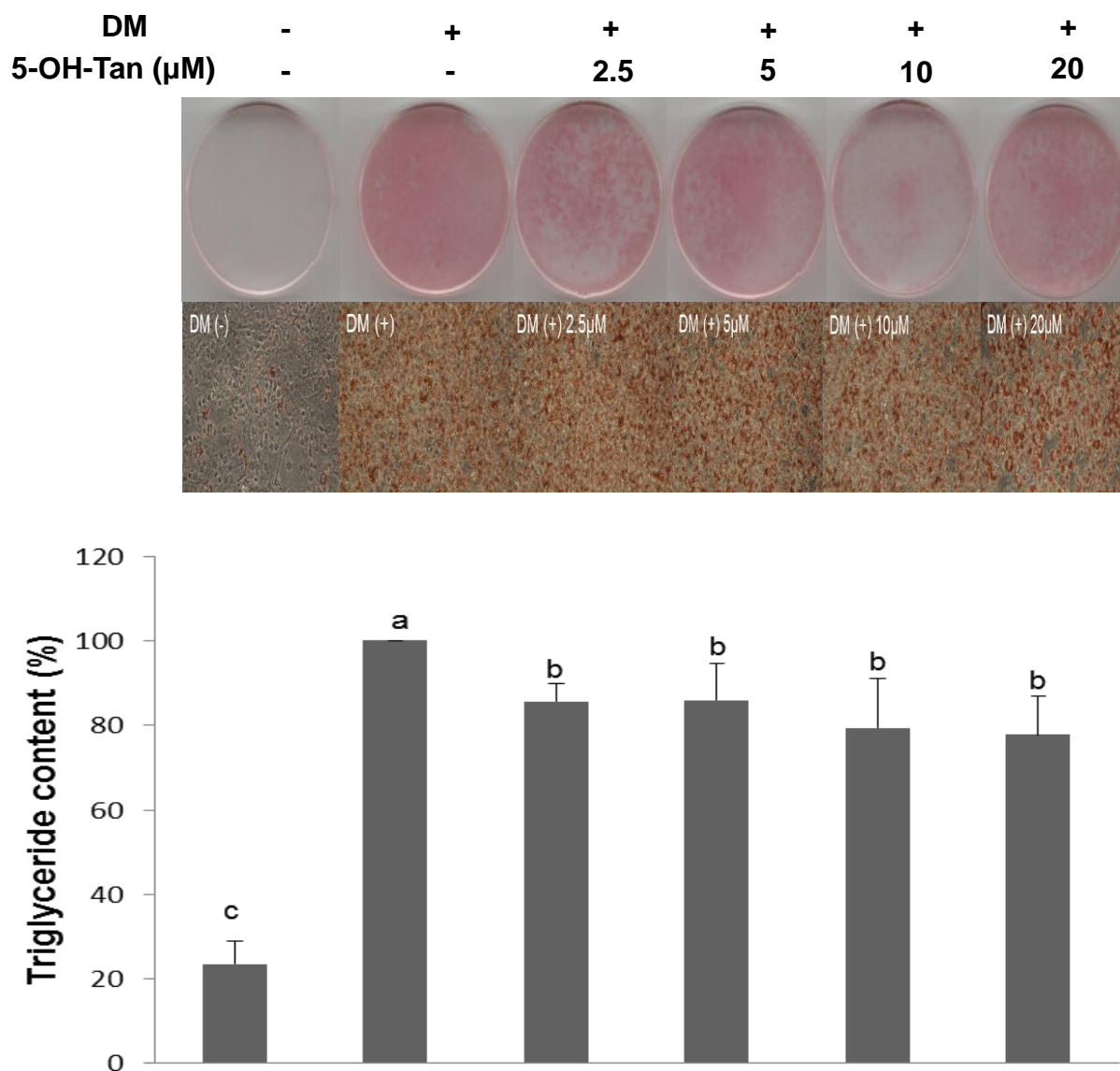


Figure 23. Effect of different concentrations of 5-demethyltangeretin (5-OH-Tan) on triglyceride content in a 3T3-L1 preadipocyte model.

DM (-): without differentiation medium; DM (+): differentiation medium containing insulin, IBMX, DEX, rosiglitazone

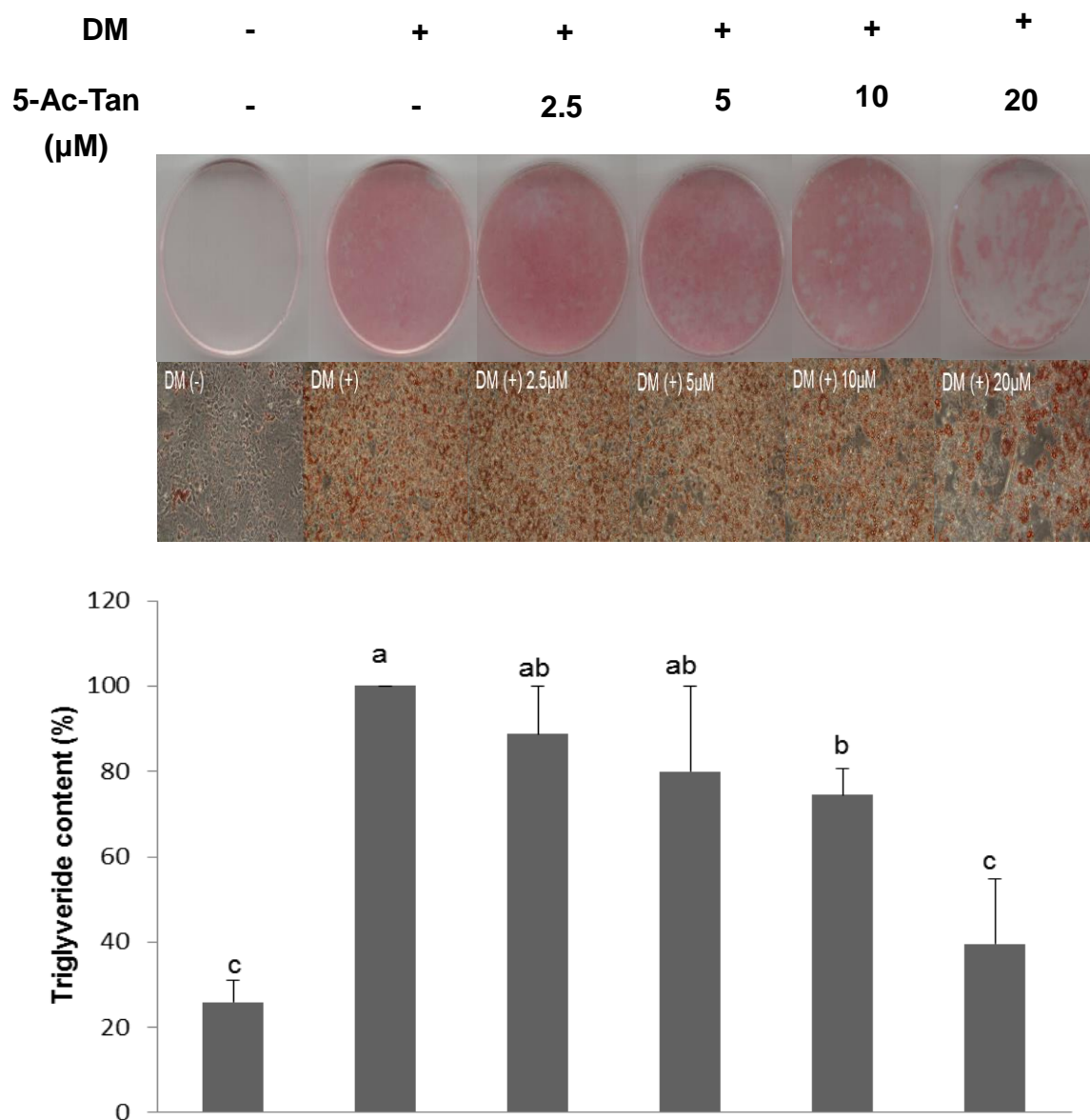


Figure 24. Effect of different concentrations of 5-acetyloxy-6,7,8,4'-tetramethoxyflavone (5-Ac-Tan) on triglyceride content in a 3T3-L1 preadipocyte model.
DM (-): without differentiation medium; DM (+): differentiation medium containing insulin, IBMX, DEX, rosiglitazone

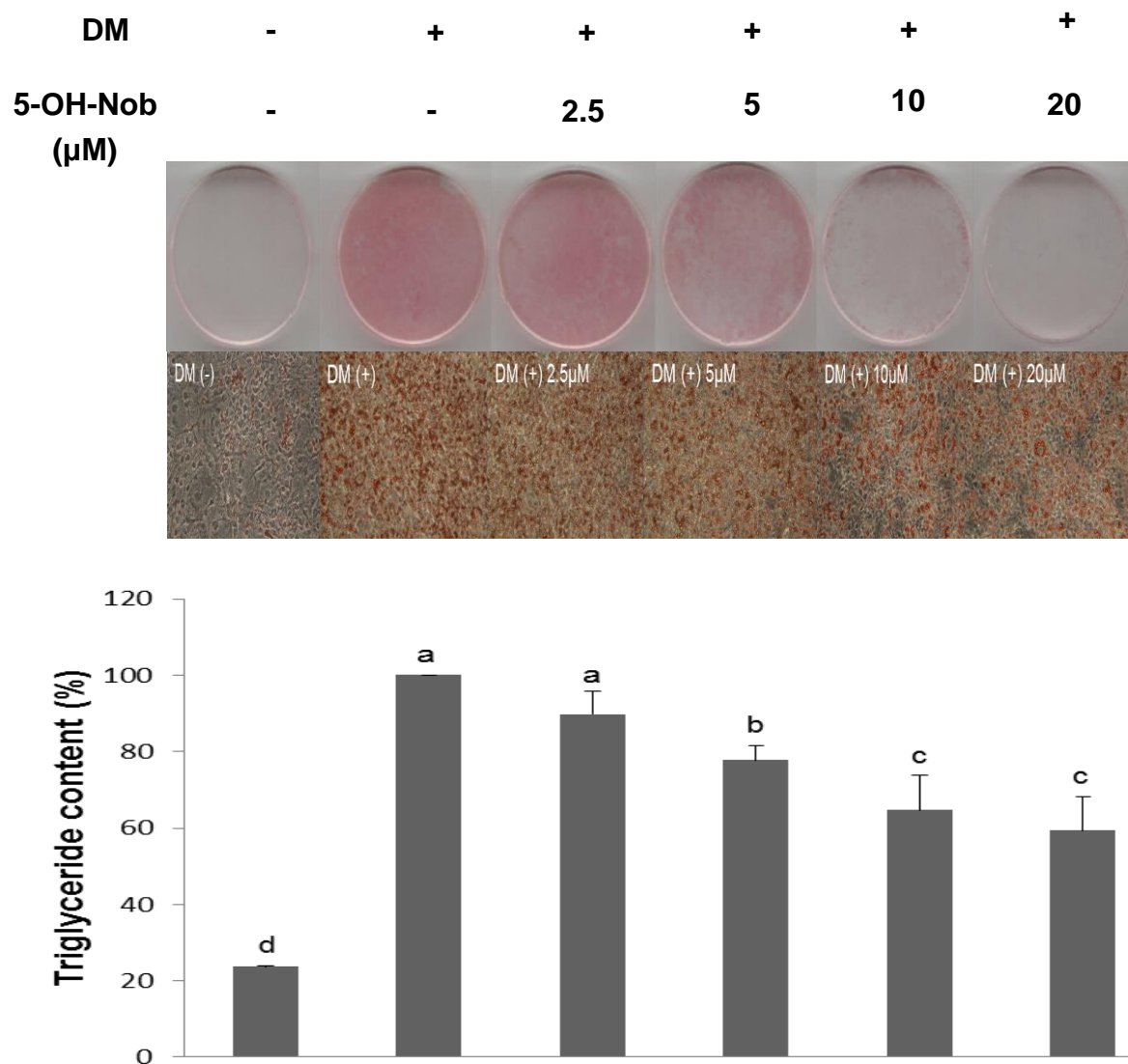


Figure 25. Effect of different concentrations of 5-demethylnobiletin (5-OH-Nob) on triglyceride content in a 3T3-L1 preadipocyte model.

DM (-): without differentiation medium; DM (+): differentiation medium containing insulin, IBMX, DEX, rosiglitazone

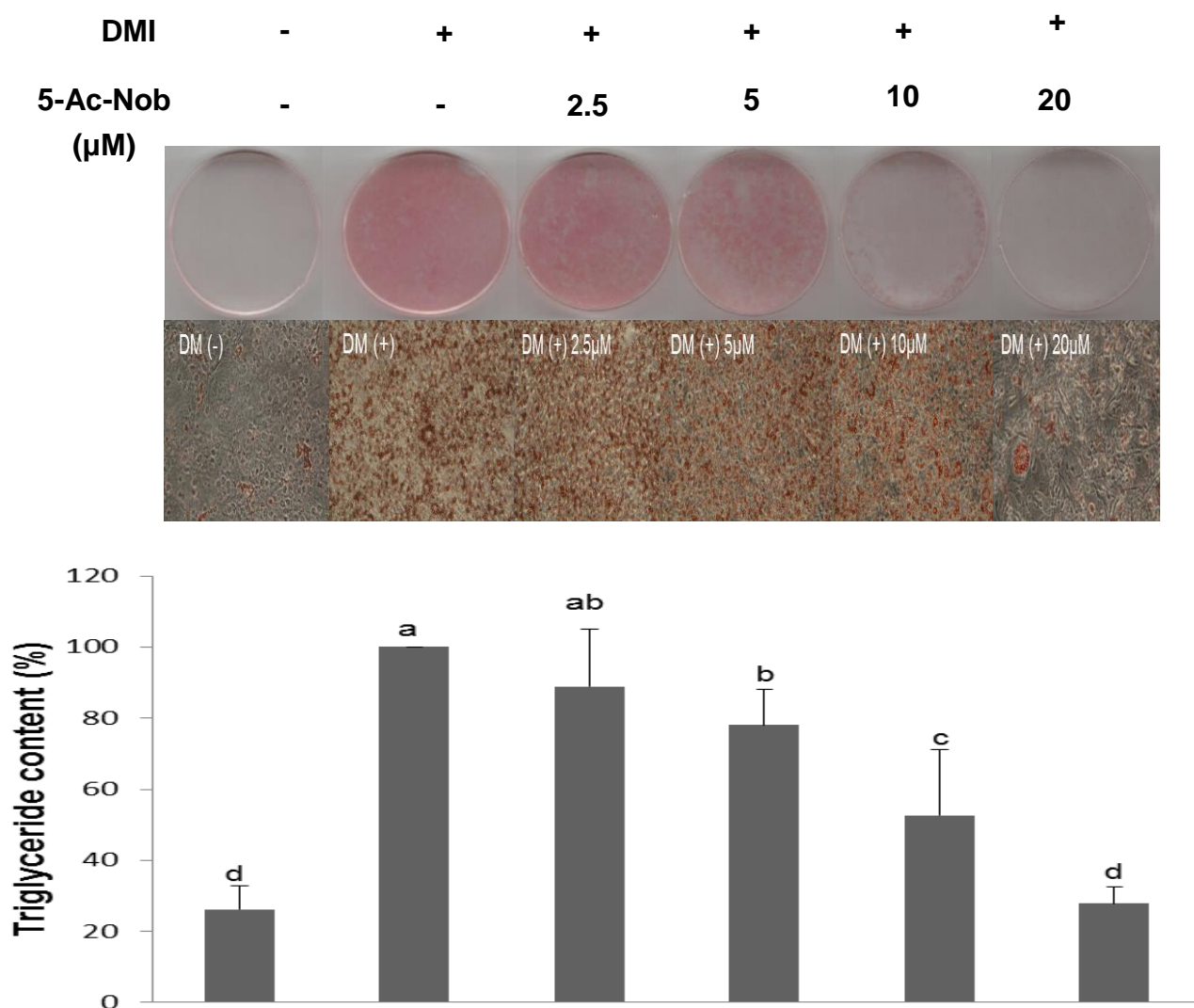


Figure 26. Effect of different concentrations of 5-acetyloxy-6,7,8,3',4'-pentamethoxyflavone (5-Ac-Nob) on triglyceride content in a 3T3-L1 preadipocyte model.

DM (-): without differentiation medium; DM (+): differentiation medium containing insulin, IBMX, DEX, rosiglitazone

6.2.3 Cell cycle analysis

According to the above results, 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob decreased lipid accumulation in the cells; therefore, we investigated their effects on cell growth during adipogenesis by analyzing the cell cycle. As shown in Figure 27, illustrating the DM (+) group, the percentage of the G0/G1 phase was 63.32%, lower than that of the DM (-) group, and the percentage of the S phase was 12.96%, higher than that of the DM (-) group. This indicated that the cells re-entered the cell cycle in the DM (+) group. When cells were treated with 2.5, 5, 10, and 20 μ M 5-OH-Tan, the cells in the G0/G1 phase decreased and those in the S phase increased, and there was no sub-G1 phase. This indicated that 5-OH-Tan did not affect the cell cycle during cell differentiation. As shown in Figure 28, illustrating the DM (+) group, the percentage of the G0/G1 phase was 54.04%, lower than that of the DM (-) group, and the percentage of the S phase was 15.31%, higher than that of the DM (-) group. This indicated that the cells re-entered the cell cycle in the DM (+) group. When cells were treated with 2.5, 5, 10, and 20 μ M 5-Ac-Tan, the cells in the G0/G1 phase decreased and those in the S phase increased. With 20 μ M 5-Ac-Tan, 10.55% of cells in the sub-G1 phase. This indicated that 5-Ac-Tan did not affect the cell cycle, but 20 μ M 5-Ac-Tan would cause apoptosis during cell differentiation. As shown in Figure 29, illustrating the DM (+) group, the percentage of the G0/G1 phase was 44.55%, lower than that of the DM (-) group, and the percentage of the S phase was 30.29%, higher

than that of the DM (-) group. This indicated that the cells re-entered the cell cycle in the DM (+) group. When cells were treated with 2.5, 5, 10, and 20 μ M 5-OH-Nob, the cells in the G0/G1 phase decreased and those in the S phase increased, and there was no sub-G1 phase. This indicated that 5-OH-Nob did not affect the cell cycle during cell differentiation. As shown in Figure 30, illustrating the DM (+) group, the percentage of the G0/G1 phase was 51.9%, lower than that of the DM (-) group, and the percentage of the S phase was 16.01%, higher than that of the DM (-) group. This indicated that the cells re-entered the cell cycle in the DM (+) group. When cells were treated with 2.5, 5, 10, and 20 μ M 5-Ac-Nob, the cells in the G0/G1 phase decreased and those in the S phase increased, and there was no sub-G1 phase. This indicated that 5-Ac-Nob did not affect the cell cycle during cell differentiation. According to the results of the cell viability and cell cycle testing, 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob would not cause any toxicity in 3T3-L1 preadipocytes except 20 μ M 5-Ac-Tan, which induced apoptosis in the cells.

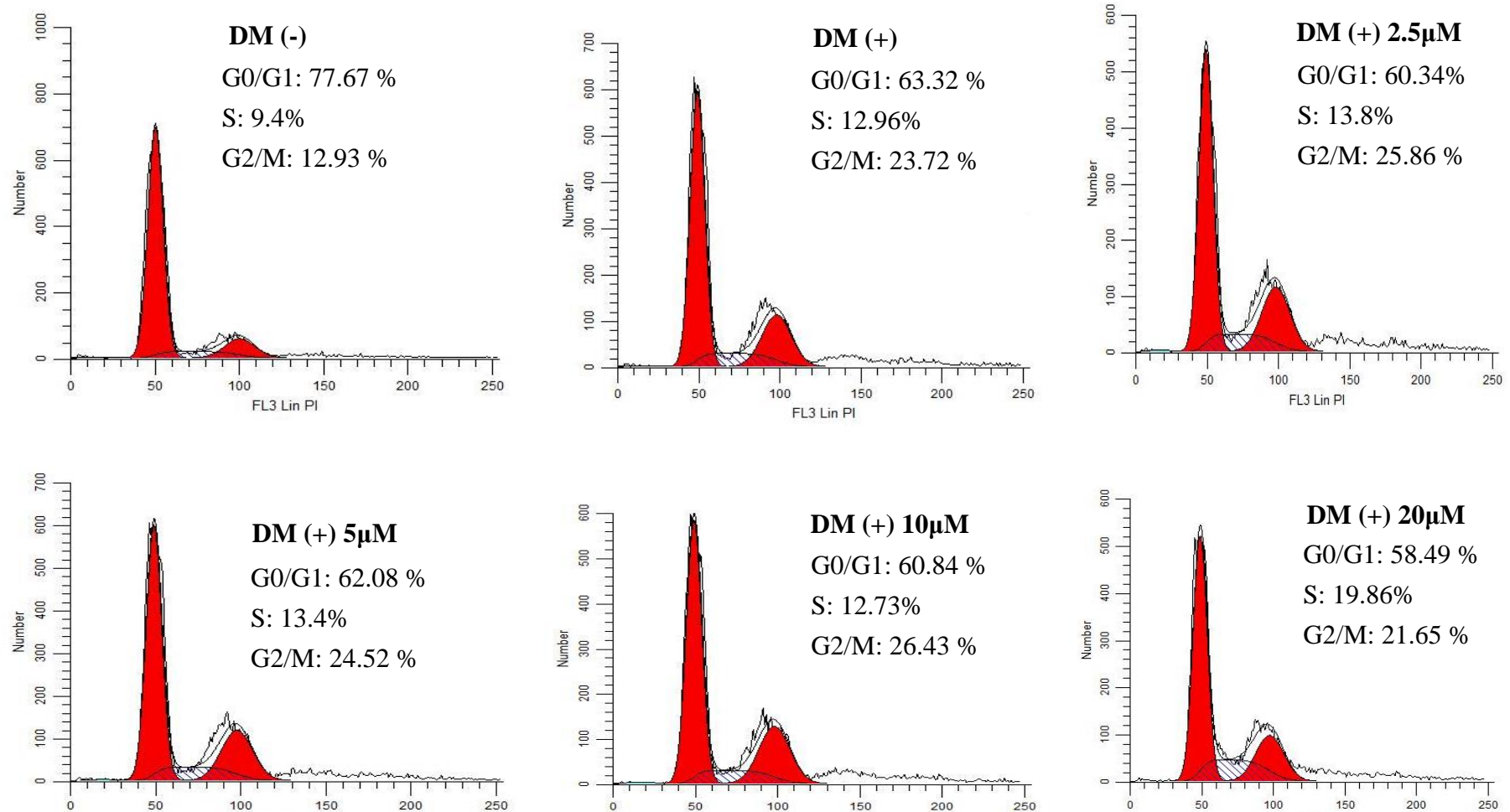


Figure 27. Effect of different concentrations of 5-demethyltangeretin (5-OH-Tan) on the cell cycle in 3T3-L1 preadipocytes for 24 hr. DM (-): without differentiation medium; DM (+): differentiation medium containing insulin, IBMX, DEX, rosiglitazone

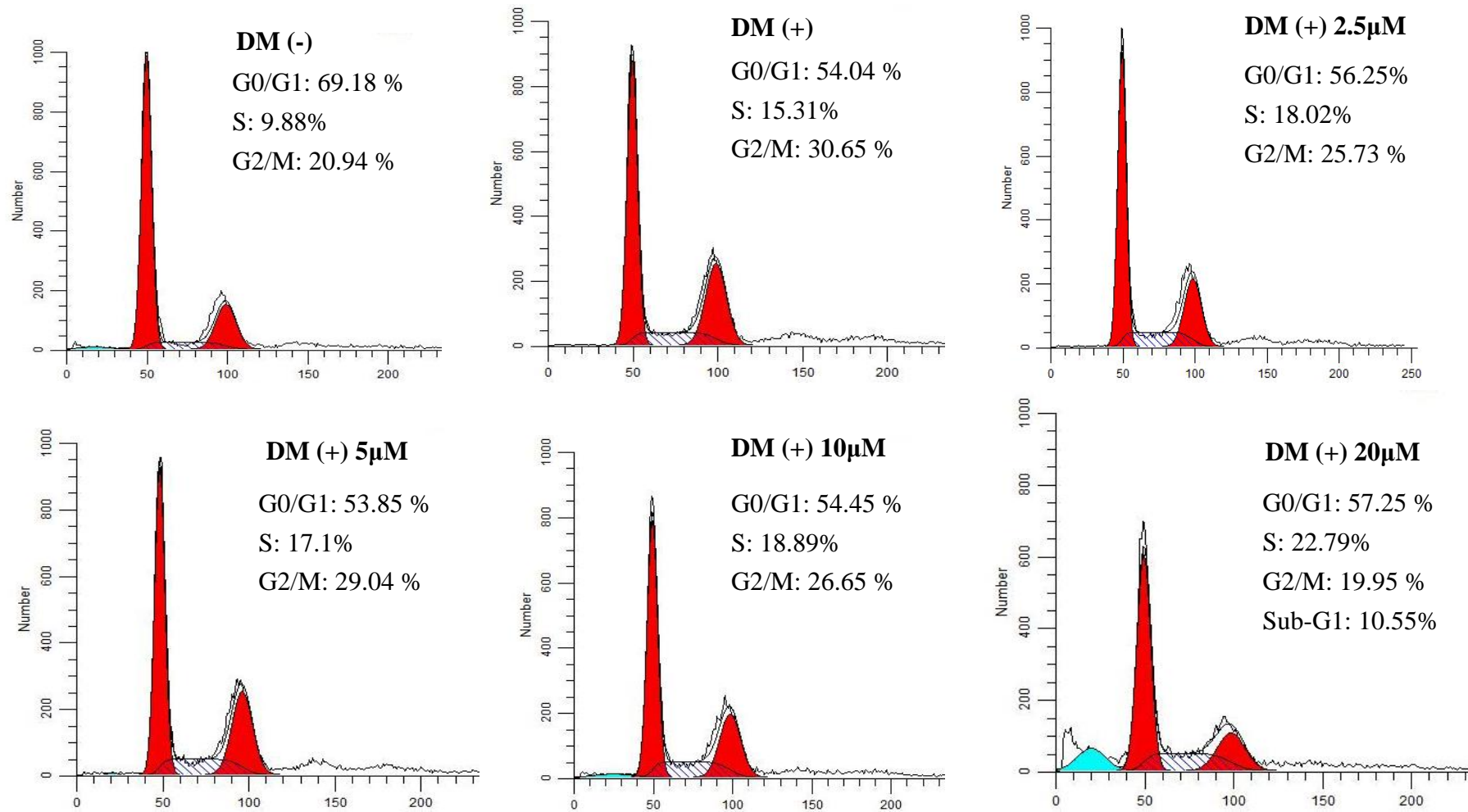


Figure 28. Effect of different concentrations of 5-acetyloxy-6,7,8,4'-tetramethoxyflavone (5-Ac-Tan) on the cell cycle in 3T3-L1 preadipocytes for 24 hr. DM (-): without differentiation medium; DM (+): differentiation medium containing insulin, IBMX, DEX,

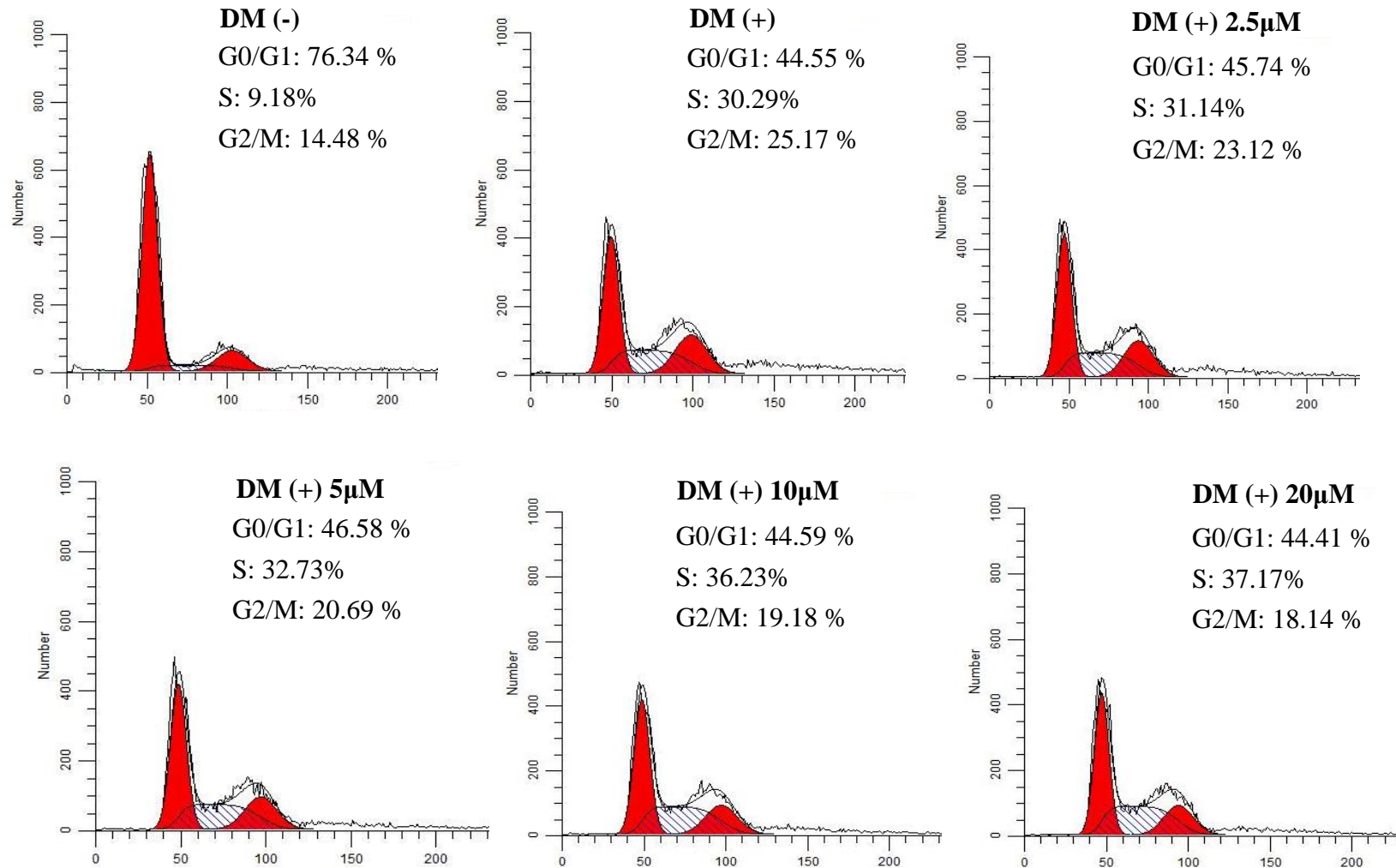


Figure 29. Effect of different concentrations of 5-demethylnobiletin (5-OH-Nob) on the cell cycle in 3T3-L1 preadipocytes for 24 hr. DM (-): without differentiation medium; DM (+): differentiation medium containing insulin, IBMX, DEX, rosiglitazone

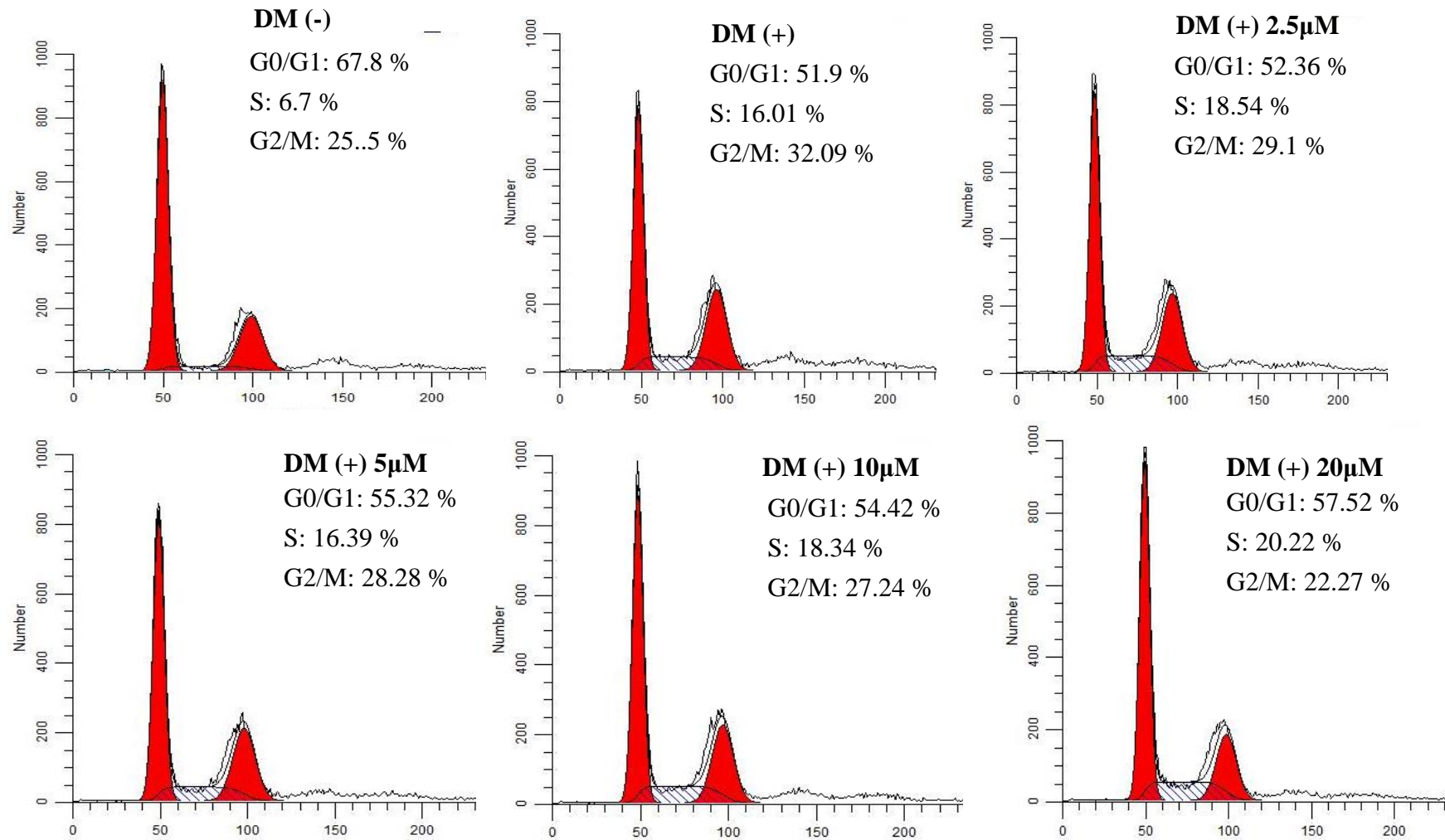


Figure 30. Effect of different concentrations of 5-acetyloxy-6,7,8,3',4'-pentamethoxyflavone (5-Ac-Nob) on the cell cycle in 3T3-L1 preadipocytes for 24 hr. DM (-): without differentiation medium; DM (+): differentiation medium containing insulin, IBMX, DEX, rosiglitazone

6.2.4 Effect of protein expression on adipogenesis

We next investigated the anti-adipogenesis effect of 5-Ac-NOB in the lipid accumulation stage. As shown in Figure 31, we found that the DM (+) group had lower LKB1, phosphorylated LKB1, and phosphorylated AMPK α protein levels than the DM (-) group. 5-Ac-Nob increased the phosphorylation of LKB1 and AMPK α protein levels compared with the DM (+) group in a dose-dependent manner. We also found that the DM (+) group had higher SREBP1, FAS, and phosphorylated ACC protein levels than the DM (-) group. 5-Ac-Nob decreased SREBP-1 and FAS protein levels, but it did not increase the phosphorylation of the ACC protein level. This indicated that 5-Ac-Nob decreased lipid accumulation by regulating LKB1 and AMPK α protein levels and then affected transcriptional factor SREBP-1 and decreased lipid synthesis protein FAS expression, but it did not affect the ACC protein level.

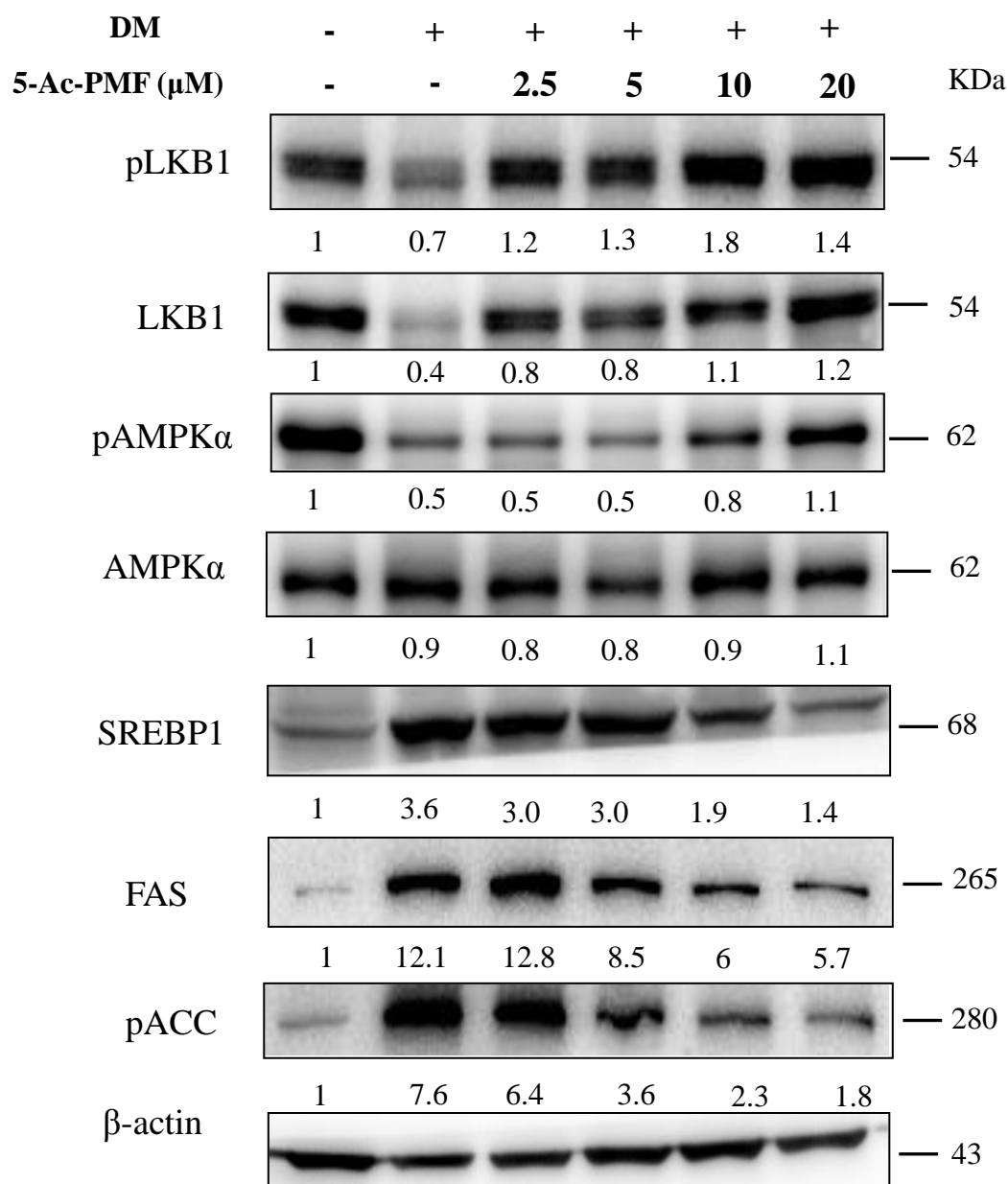


Figure 31. Effect of 5-Ac-Nob on adipogenesis-related protein levels in 3T3-L1.

6.3 Anti-adipogenesis in an HFD-induced obesity animal model

6.3.1 Effect of 5-Ac-Nob on body weight

According to the above results, 5-Ac-Nob decreased lipid accumulation in the 3T3-L1 preadipocyte model; therefore, we further investigate its anti-adipogenesis in the animals. We fed the mice a diet with 45% calories from fat for 13 weeks and oral gavage with 25 and 50 mg/kg of 5-Ac-Nob. After 13 weeks, the body weight of the HFD group was 29.6 g, higher than the body weight of the control group, 25.5 g. At the end of the experiment, the body weight of the LAN and HAN groups was 27 and 27.5 g respectively, lower than the HFD group (Figure 32).

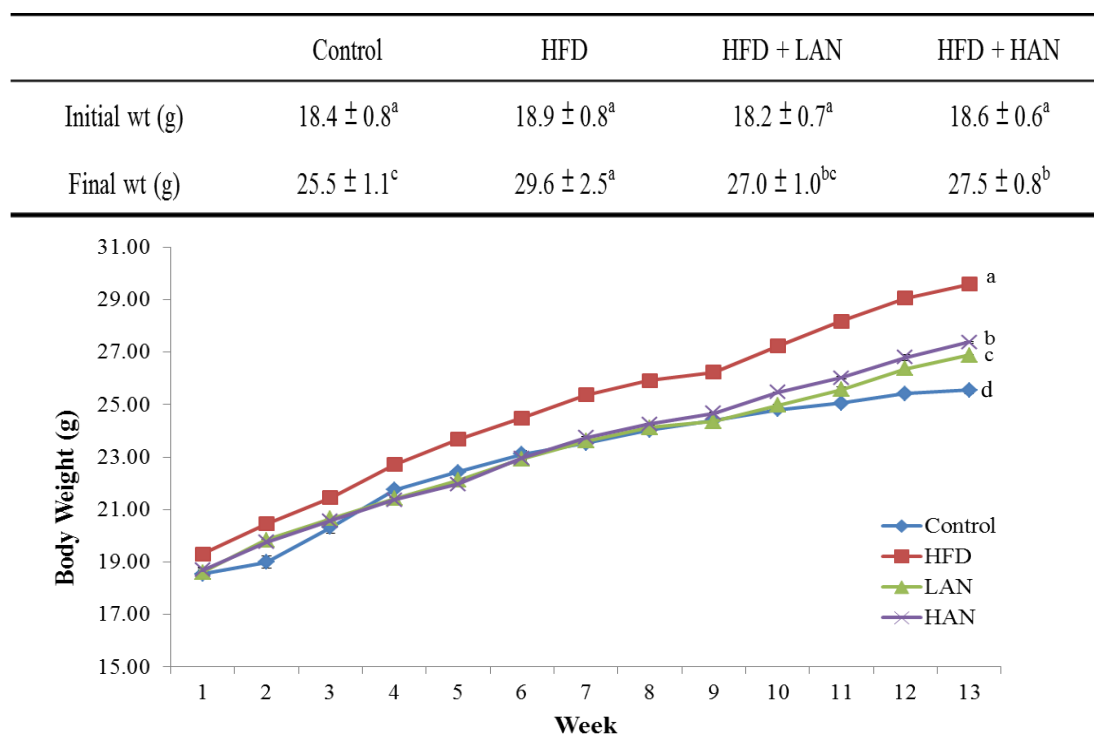


Figure 32. Effect of 5-Ac-Nob on body weight. Data are expressed as mean ± SD (n=8) and analyzed statistically using one-way ANOVA and Duncan's test. Different letters (a-b) represent statistically significant differences among treatments, $p < 0.05$.

6.3.2 Effect of 5-Ac-Nob on food intake

During the experiment, we recorded food intake every day and found that the control group had higher food intake than the HFD, LAN, and HAN groups. There was no significant difference in food intake among the HFD, LAN, and HAN groups (Figure 33).

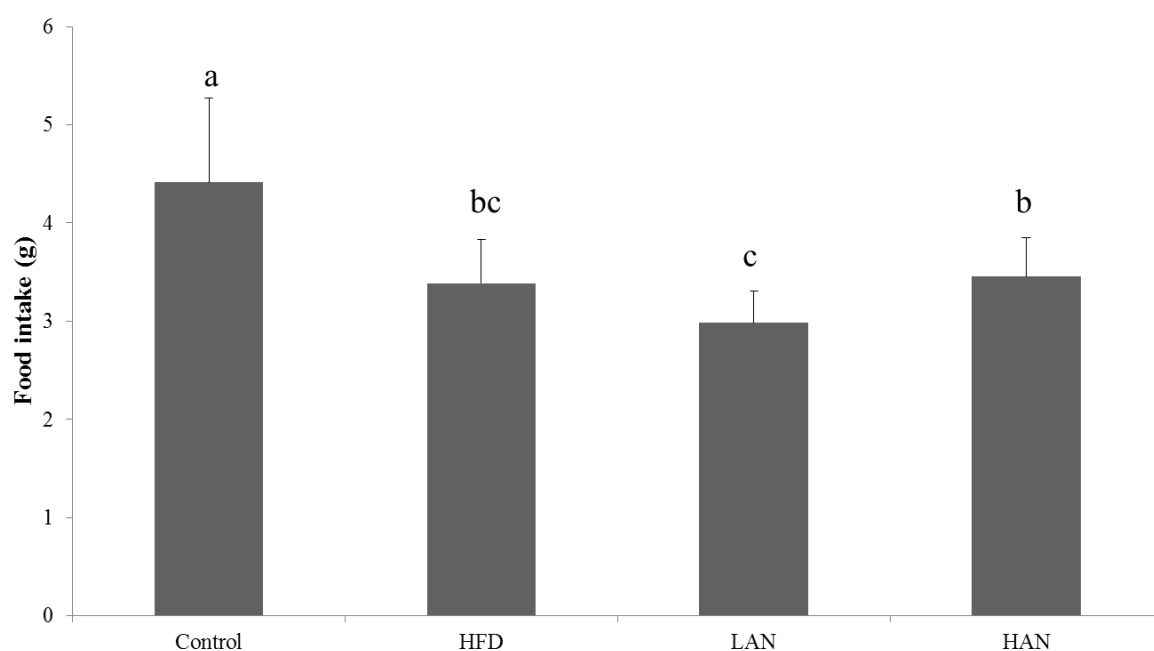


Figure 33. Effect of 5-Ac-Nob on food intake. Data are expressed as mean \pm SD (n=8) and analyzed statistically using one-way ANOVA and Duncan's test. Different letters (a-b) represent statistically significant differences among treatments, $p < 0.05$.

6.3.3 Effect of 5-Ac-Nob on organ weight

At the end of the experiment, we collected live kidney and spleen and then weighed them. The organ weight did not show any significant difference among the groups (Table 8).

Table 7. Effect of 5-Ac-Nob on Organ weight

| | Control | HFD | HFD + LAN | HFD + HAN |
|---------------|----------------------|----------------------|-------------------|-------------------|
| Liver wt (g) | 1.25 ± 0.05^{ab} | 1.25 ± 0.05^{ab} | 1.26 ± 0.06^a | 1.20 ± 0.04^b |
| Kidney wt (g) | 0.42 ± 0.01^a | 0.42 ± 0.01^a | 0.41 ± 0.02^a | 0.42 ± 0.02^a |
| Spleen wt (g) | 0.07 ± 0.01^a | 0.06 ± 0.01^a | 0.06 ± 0.01^a | 0.06 ± 0.01^a |

Data are expressed as mean \pm SD (n=8) and analyzed statistically using one-way ANOVA and Duncan's test. Different letters (a-b) represent statistically significant differences among treatments, $p < 0.05$.

6.3.4 Effect of 5-Ac-Nob on white adipose tissue (WAT) – intra-abdominal fat weight

At the end of the experiment, we collected and weighed perigonadal fat, retroperitoneal fat, and mesenteric fat. The HFD group had higher perigonadal fat, retroperitoneal fat, and mesenteric fat weights than the control group. In the LAN and HAN groups, the intra-abdominal fat weights were lower than those of the HFD group (Table 9).

Table 8. Effect of 5-Ac-Nob on white fat tissue weight

| | Control | HFD | HFD + LAN | HFD + HAN |
|----------------------------|-------------------|-------------------|-------------------|-------------------|
| Perigonadal fat wt (g) | 0.32 ± 0.04^d | 0.86 ± 0.09^a | 0.68 ± 0.06^c | 0.75 ± 0.06^b |
| Retroperitoneal fat wt (g) | 0.06 ± 0.01^c | 0.27 ± 0.05^a | 0.23 ± 0.02^b | 0.24 ± 0.02^b |
| Mesenteric fat wt (g) | 0.27 ± 0.06^b | 0.39 ± 0.09^a | 0.31 ± 0.04^b | 0.30 ± 0.04^b |
| WAT wt (g) | 0.64 ± 0.06^c | 1.52 ± 0.14^a | 1.23 ± 0.07^b | 1.29 ± 0.06^b |

Data are expressed as mean \pm SD (n=8) and analyzed statistically using one-way ANOVA and Duncan's test. Different letters (a-b) represent statistically significant differences among treatments, $p < 0.05$.

6.3.5 Effect of 5-Ac-Nob on serum biochemistry data

According to the Table 9, the GOT data did not show any difference among the four groups. The HFD and HAN groups had lower GPT levels than the control and LAN groups. The TG level of the HAN group was 82.1 mg/dl, lower than the 97.1 mg/dl of the HFD group. The total cholesterol level of the HAN group was 117.4 mg/dl, lower than the 134.4 mg/dl of the HFD group (Table 10).

Table 9. GOT, GPT value in Male C57BL/6 mice¹⁴⁰

| Intracadiac | | | | | | |
|-------------|----------|---------|------|-----|-----|-----|
| Parameter | Units | p value | Mean | Min | Max | SD |
| GOT | IU/liter | | 82 | 51 | 232 | 45 |
| GPT | IU/liter | 0.5954 | 38 | 30 | 51 | 3.6 |

Table 10. Effect of 5-Ac-Nob on serum biochemistry data

| | Control | HFD | HFD + LAN | HFD + HAN |
|---------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| GOT | 116.6 \pm 29.3 ^a | 110.6 \pm 17.4 ^a | 124.8 \pm 38.3 ^a | 106.3 \pm 26.3 ^a |
| GPT | 41.4 \pm 3.0 ^a | 27.3 \pm 2.2 ^b | 38.8 \pm 18.3 ^a | 27.6 \pm 1.9 ^b |
| TG | 74.6 \pm 5.3 ^b | 97.1 \pm 13.8 ^a | 94.6 \pm 13.2 ^a | 82.1 \pm 7.1 ^b |
| T-Cholesterol | 69.3 \pm 4.8 ^c | 134.4 \pm 8.1 ^a | 132.8 \pm 7.3 ^a | 117.4 \pm 11.0 ^b |

Data are expressed as mean \pm SD (n=8) and analyzed statistically using one-way ANOVA and Duncan's test. Different letters (a-b) represent statistically significant differences among treatments, $p < 0.05$.

6.3.6 Histopathological examination of the liver

According to Table 11, there was no toxicity or fatty change in the liver.

Table 11. Pathology – individual liver score of mice in the high-fat diet-induced fatty liver

| Group/ organ | Histopathological findings | Animal code | | | | | | | | | | | | | | | |
|-----------------|-------------------------------------|-------------|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|
| | | C | | | | | | | | | | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Liver | | | | | | | | | | | | | | | | | |
| | Fatty change, micro-vesicles, focal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | |
| | Fatty change, macro-vesicles, focal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | |
| | Necrosis, focal | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | | | | | | | | |
| Group/ organ | Histopathological findings | Animal code | | | | | | | | | | | | | | | |
| | | HFD | | | | | | | | | | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Liver | | | | | | | | | | | | | | | | | |
| | Fatty change, micro-vesicles, focal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | |
| | Fatty change, macro-vesicles, focal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | |
| | Necrosis, focal | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | | | | | | | | |
| Group/ organ | Histopathological findings | Animal code | | | | | | | | | | | | | | | |
| | | HFD+LAN | | | | | | | | | | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Liver | | | | | | | | | | | | | | | | | |
| | Fatty change, micro-vesicles, focal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | |
| | Fatty change, macro-vesicles, focal | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | | | | | | | | |
| | Necrosis, focal | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | | | | | | | | |
| Group/ organ | Histopathological findings | Animal code | | | | | | | | | | | | | | | |
| | | HFD+HAN | | | | | | | | | | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Liver | | | | | | | | | | | | | | | | | |
| | Fatty change, micro-vesicles, focal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | |
| | Fatty change, macro-vesicles, focal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | |
| | Necrosis, focal | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | |

N: no tissue available. 0: No effect.

¹: Degree of lesions was graded from one to five depending on severity: 1 = minimal (< 1%); 2 = slight (1-25%); 3 = moderate (26-50%); 4 = moderate/severe (51-75%); 5 = severe/high (76-100%).

6.3.7 Liver TG

As shown in Figure 34, the TG level in the liver was 23.5, 74.8, 68.5, and 49.6 mg/dl in the control, HFD, LAN, and HAN groups, respectively. The HAN group showed decreased TG levels in the liver compared with the HFD group.

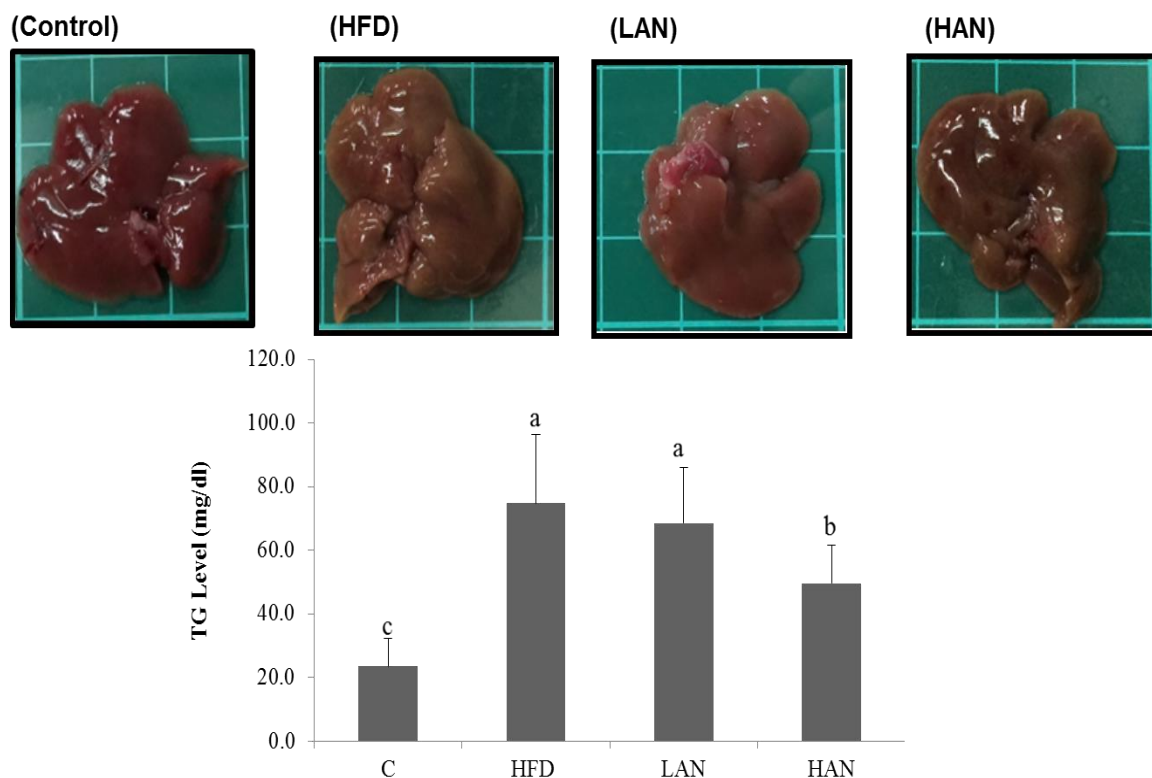


Figure 34. Effect of 5-Ac-Nob on liver TG level is expressed as mean \pm SD (n=8) and analyzed statistically using one-way ANOVA and Duncan's test. Different letters (a-b) represent statistically significant differences among treatments, $p < 0.05$.

6.3.8 Effect of protein expression in the liver

As shown in Figure 35, we found that the HFD group had lower phosphorylated LKB1 and phosphorylated AMPK α protein levels than the control group. The HAN group showed an increased phosphorylation of LKB1 and AMPK α protein levels compared with the HFD group. We also found that the HFD group had lower phosphorylated ACC protein levels than the control group. The HAN group showed

increased phosphorylation of ACC protein levels compared with the HFD group. The HFD group had lower SREBP1 and FAS protein levels than the control and LAN groups. The LAN and HAN groups did not show decreased SREBP1 and FAS protein levels. This indicated that the HAN group had decreased lipid accumulation in the liver because of increased phosphorylated LKB1 and AMPK α protein levels and decreased ACC lipid synthesis protein expression.

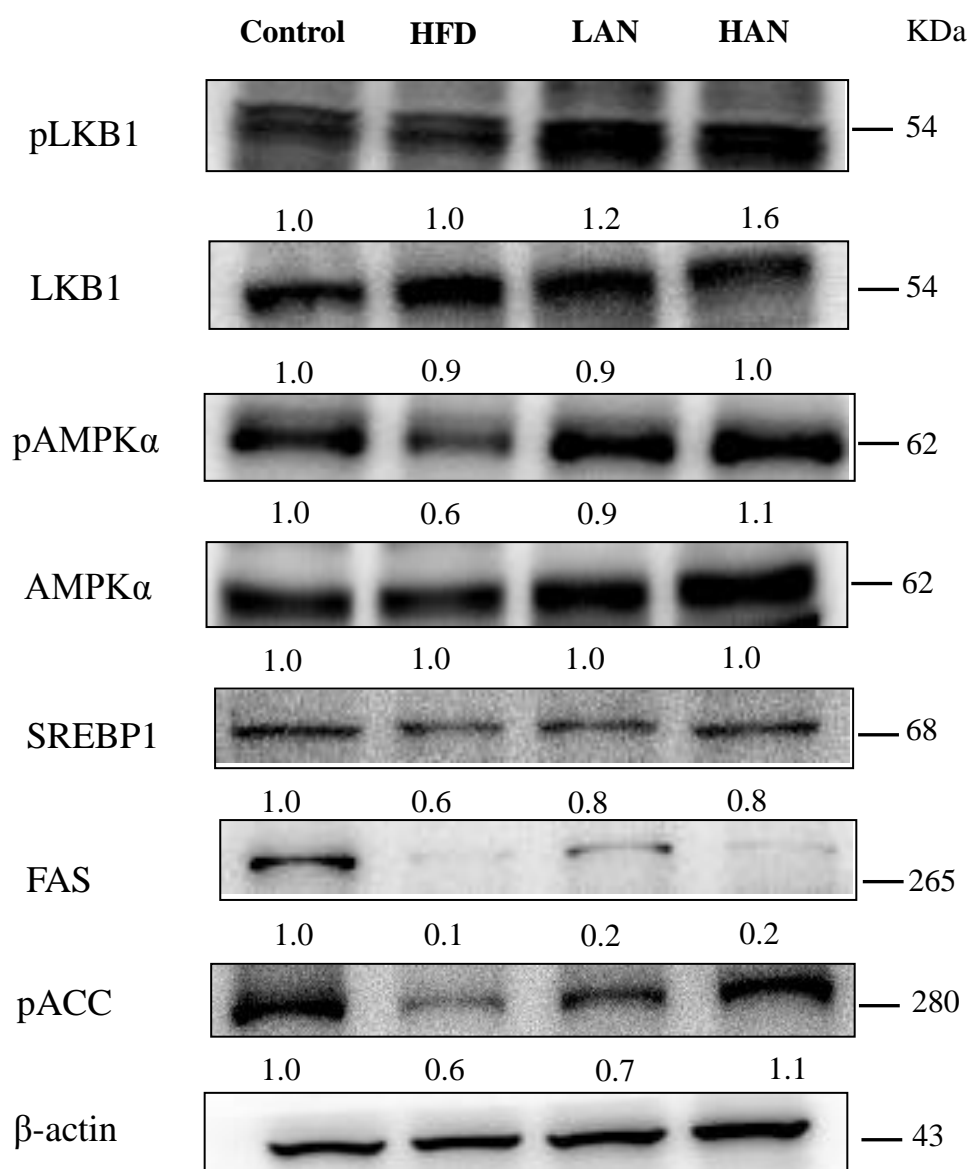


Figure 35. Effect of 5-Ac-Nob on lipogenesis-related protein levels in an HFD-induced obesity model

6.4 Level of nobiletin, 5-demethylnobiletin and 5-acetyloxy-6,7,8,3',4'-pentamethoxyflavone in mouse plasma

The results showed that nobiletin appeared in the plasma after the mouse was given 5-OH-Nob within 120 minutes, but not obviously in the 5-Ac-Nob-fed mouse (Figure 36 & 39). 5-OH-Nob also appeared in 5-OH-Nob-fed mouse within 120 minutes and the concentration of 5-OH-Nob in the 5-Ac-Nob-fed mouse was higher than the 5-OH-Nob -fed mouse at 120 minutes (Figure 37 & 40). However, the concentration of 5-Ac-Nob in plasma was relatively low even 5-Ac-Nob was directly spiked into the plasma (Figure 38 & 41).

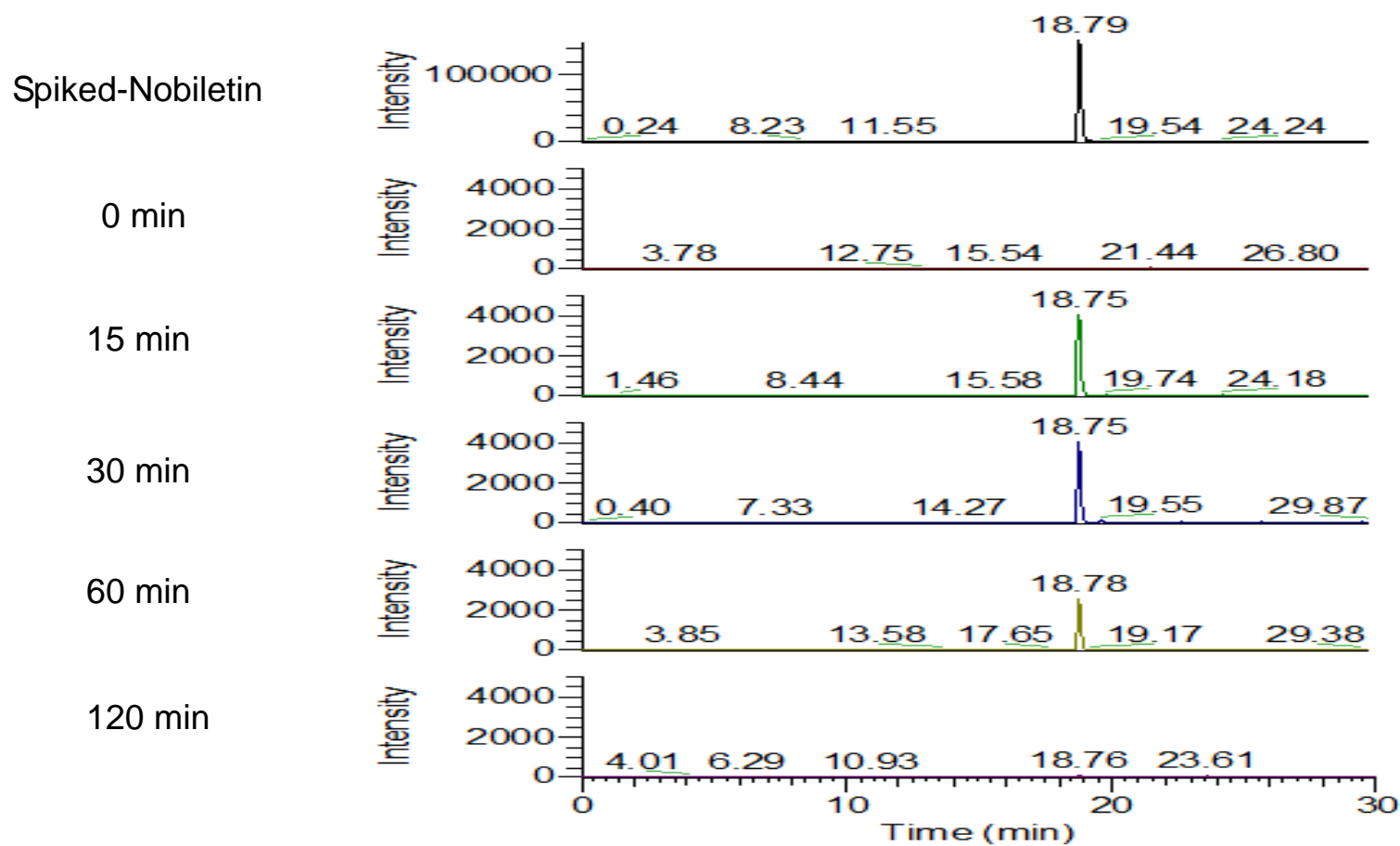


Figure 36. Time-dependent changes of nobiletin concentration in plasma after oral administration of 5-OH-Nob

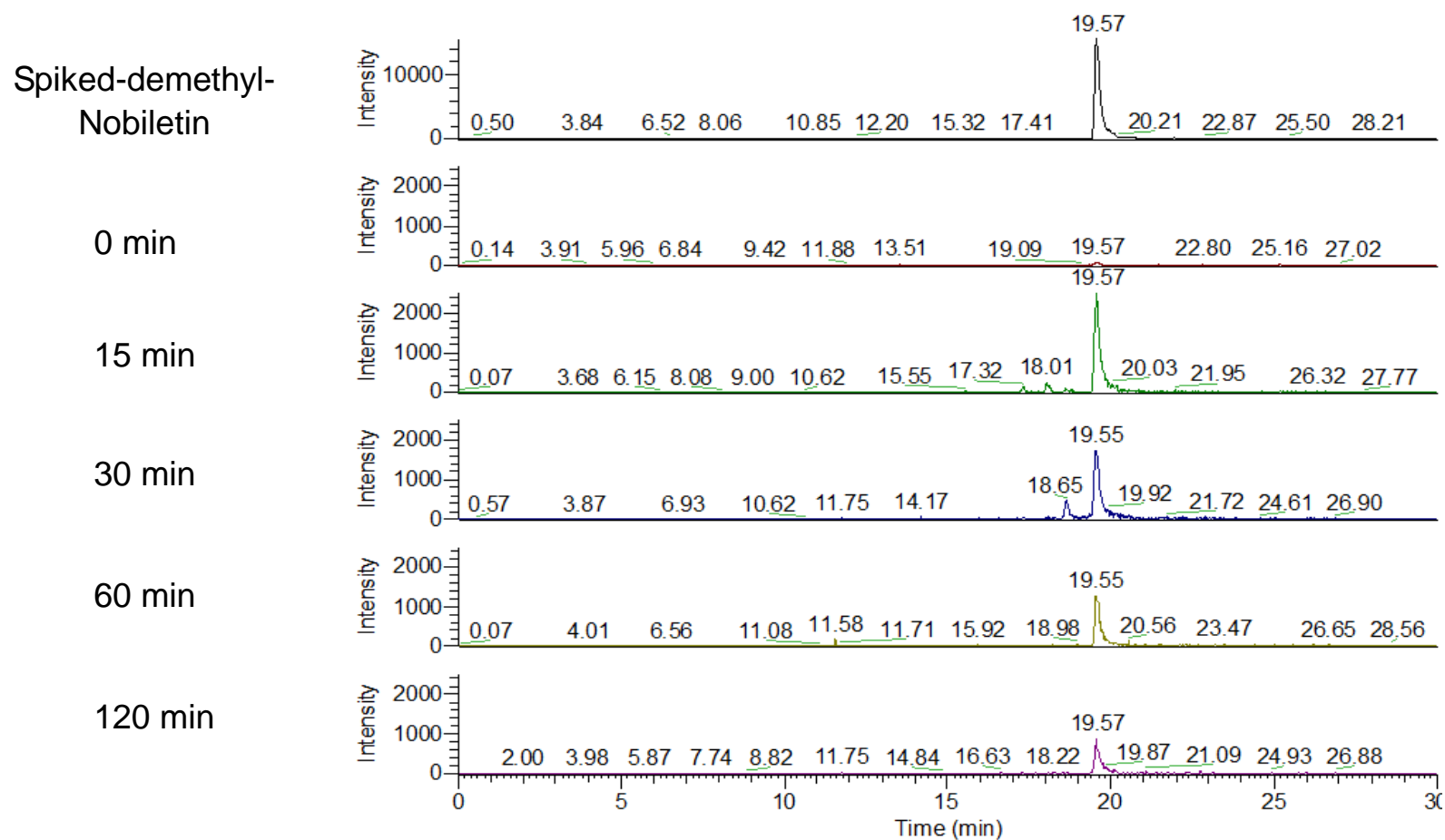


Figure 37. Time-dependent changes of 5-demethyl nobiletin concentration in plasma after oral administration of 5-OH-Nob

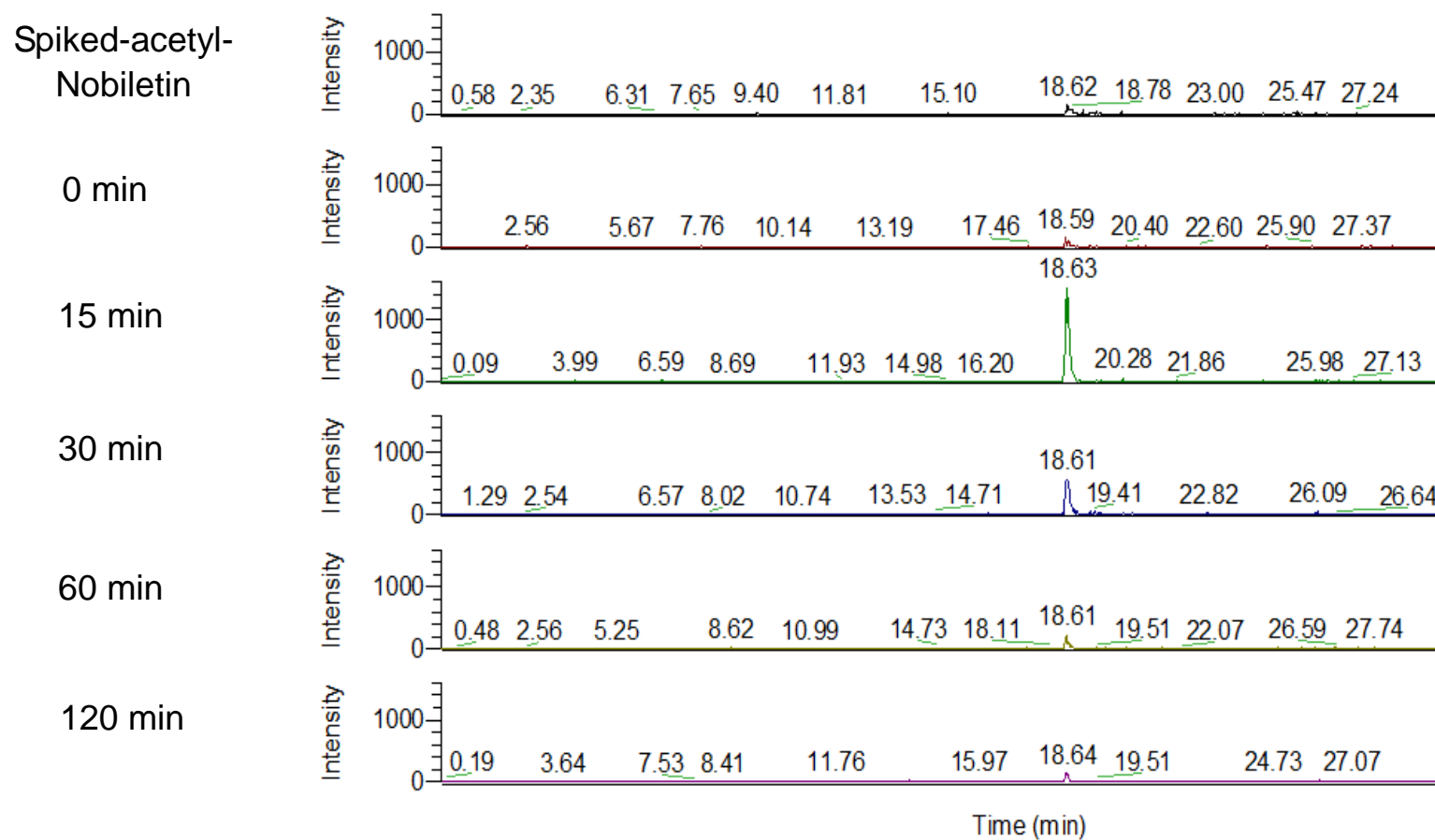


Figure 38. Time-dependent changes of 5-Ac-Nob concentration in plasma after oral administration of 5-OH-Nob

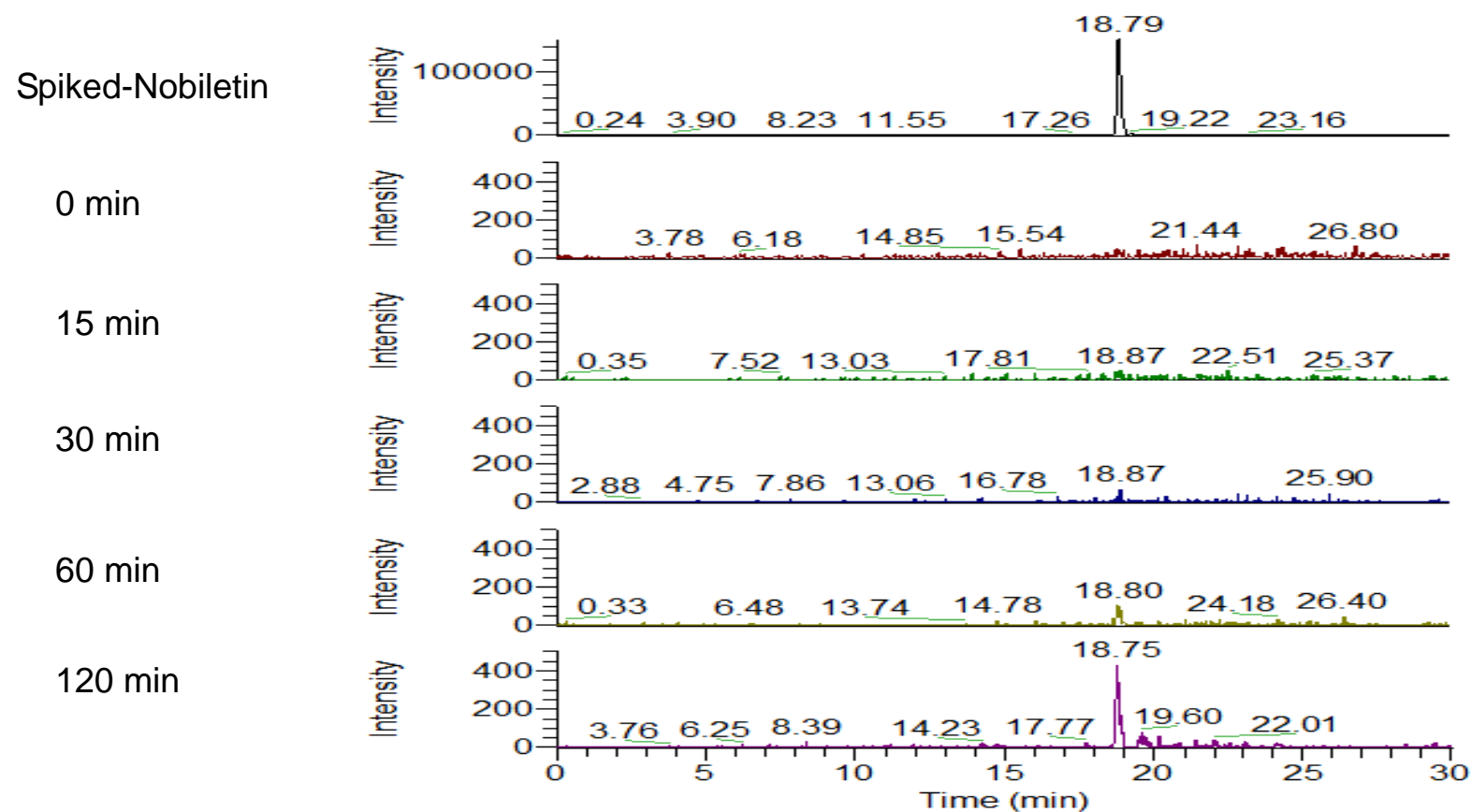


Figure 39. Time-dependent changes of nobiletin concentration in plasma after oral administration of 5-Ac-Nob

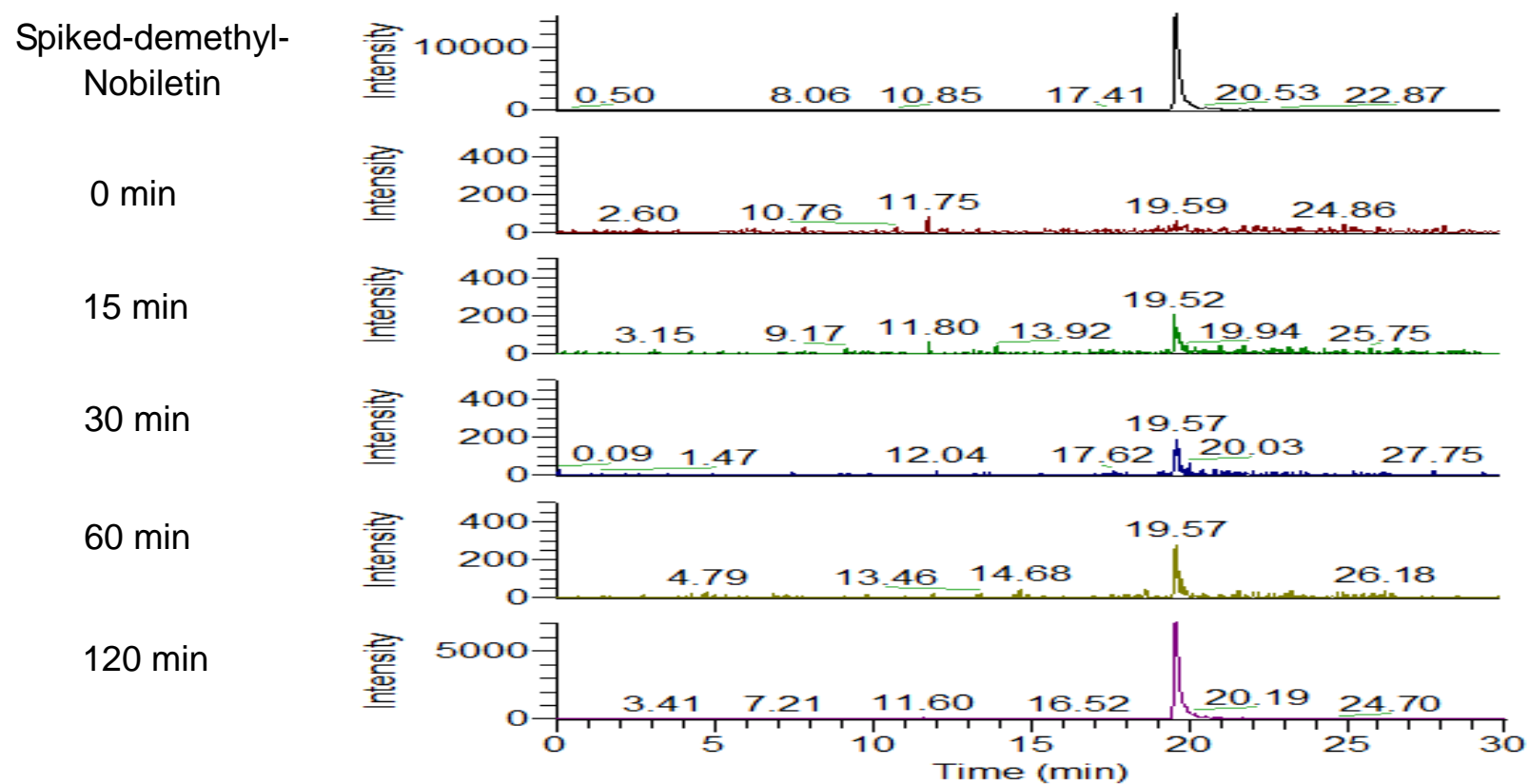


Figure 40. Time-dependent changes of 5-demethyl nobiletin concentration in plasma after oral administration of 5-Ac-Nob

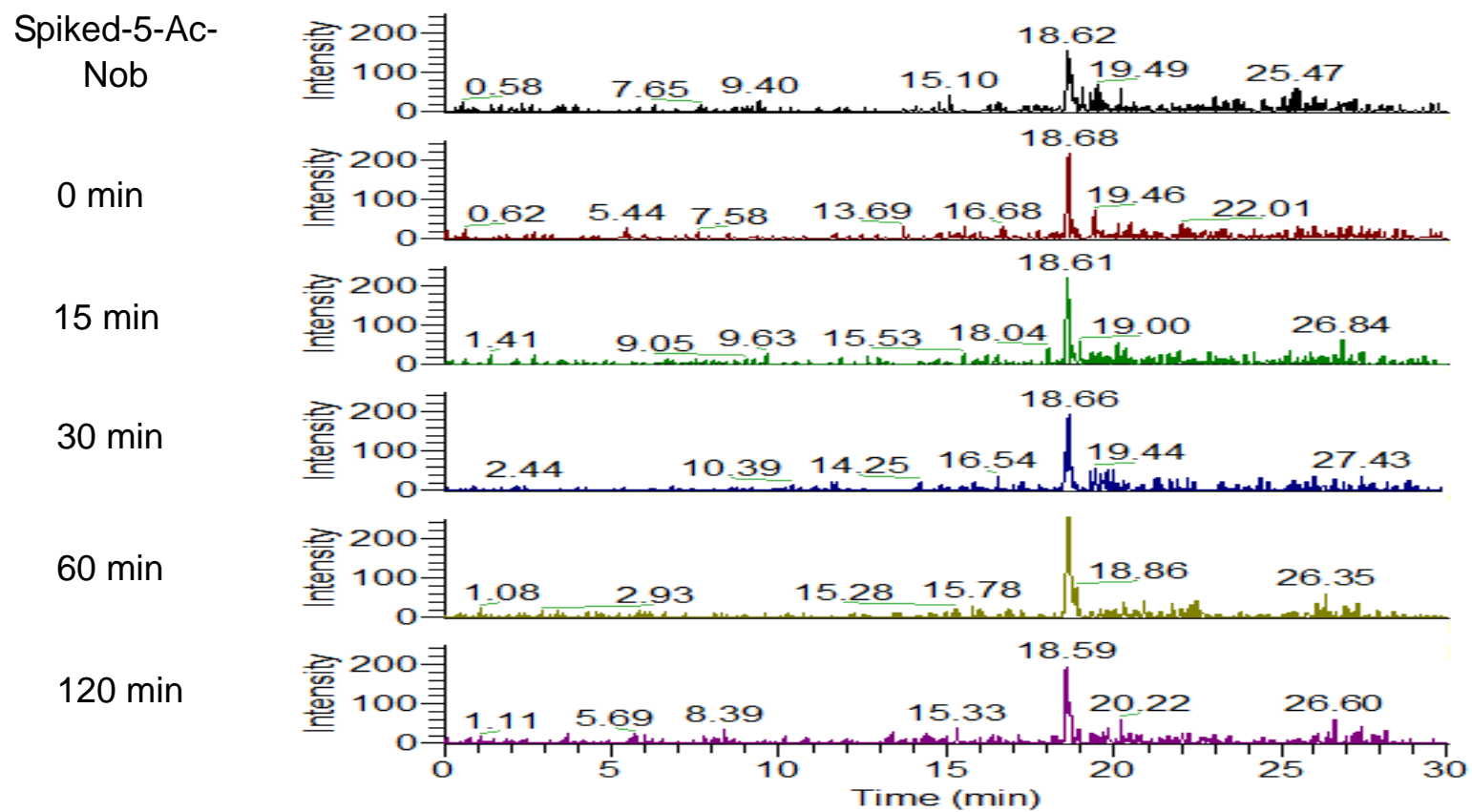


Figure 41. Time-dependent changes of 5-Ac Nob concentration in plasma after oral administration of 5-Ac-Nob

CHAPTER 7. DISCUSSION

More and more research has shown that decreased adipogenesis in the adipocytes could help control body weight. Some plant extracts such as raspberry ketones and citrus peel extract, PMFs and HPMFs, have been proven to regulate lipid metabolism in 3T3-L1 and in HFD-induced obesity animal models.^{8, 10, 121} However, PMFs and HPMFs have poor aqueous solubility, which leads to low oral bioavailability.^{104, 106} In the current study, we used the prodrug concept to make a derivative that can release active parent molecules by enzymatic and/or chemical transformation *in vivo* to enhance its oral bioavailability efficiency.¹²⁷ We used chemical modification from nobiletin and tangeretin to obtain 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob in this study (Figure 22). We need to measure our studied compounds would not cause any cytotoxicity in the cell by using LDH assay. The results of LDH assay showed that there were no significant differences among different concentration in each group (Table 6). A previous study showed that nobiletin and tangeretin could decrease triacylglycerol content by around 80%, and the concentrations were around 64 to 100 μ M in 3T3-L1 cells.¹¹ In the current study, we found that 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob decreased 20-70 percentage of triacylglycerol content at concentrations of 10 and 20 μ M. Demethylated and

acetylated forms of nobiletin and tangeretin could be more efficiency than their parent compounds, nobiletin and tangeretin. Moreover, 20 μ M 5-Ac-Nob could decreased more triacylglycerol content than 5-OH-Tan, 5-Ac-Tan and 5-OH-Nob (Figured 23-26). It seems that acetylation of nobiletin could be a method to improve its poor oral bioavailability. Adipogenesis is the process by which preadipocytes become functional mature adipocytes and this process include growth arrest, mitotic clonal expansion, and terminal phase differentiation, regulating with different genes, transcriptional factors or enzyme.^{40, 50} We found that 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob decreased lipid accumulation in 3T3-L1 cells; therefore, we wanted to know at which phase they affect adipogenesis. First, we analyzed their effect on the growth arrest phase to detect cell cycle by flow cytometry. After cells were stimulated by DM [DM (+) group], the cell would re-enter the cell cycle from G0/G1 phase proceed to S phase and they still underwent the adipogenesis process, compared with the cell were not stimulated with DM [DM (-) group]. We found that 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob let the cells proceed to the S phase (Figures 27-30). There was one exception: 20 μ M 5-Ac-Tan induced apoptosis during cell differentiation. Overall, all the studied compounds their anti-adipogenesis ability were not by affecting the growth arrest stage during adipogenesis. According to the cell viability and flow cytometry results,

5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob in different concentrations did not cause cytotoxicity and apoptosis in the 3T3-L1 cells except 20 μ M 5-Ac-Tan. Lai et al. showed that an HPMF mixture reduced triacylglycerol content by downregulating the PPAR γ and SREBP1c transcriptional factors and then affected their downstream FAS and ACC protein expression.¹⁰ In the current study, 5-Ac-Nob reduced lipids in the cells more than other compounds and did not cause toxicity or apoptosis. We further investigated its anti-adipogenesis-related molecular mechanism in 3T3-L1 preadipocyte model. Generally, the activation of SREBPs involves a two-step cleavage process; after it has been cleaved, its nuclear form (68kd) is released from the endoplasmic reticulum membrane and then to the promoters of target genes to activate transcription.^{59, 60} We found that 5-Ac-Nob decreased the active form of SREBP-1 protein levels at 68 kd in a dose-dependent manner (Figure 31). Base on the cell cycle and active form of SREBP-1 protein level results showed that 5-Ac-Nob reduced lipid accumulation, possibly at the terminal phase differentiation. In the terminal phase differentiation, lipogenesis increases greatly regulated by the protein and mRNA expression of triacylglycerol metabolism-related enzymes such as acetyl-CoA carboxylase (ACC), stearyl-CoA desaturase, fatty acid synthase (FAS), and glyceraldehyde-3-phosphatedehydrogenase increased 10- to 100-fold.^{51, 141, 142} We found that 5-Ac-Nob decreased FAS protein levels

in a dose-dependent manner. However, 5-Ac-Nob did not decrease the inactive form of ACC when it was in the phosphorylation form. All these results showed that 5-Ac-Nob decreased lipid accumulation by decreasing the SREBP-1 and FAS protein levels. Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) plays a key role in energy homeostasis. Liver kinase B1 (LKB1) is an upstream of AMPK, and it also activates AMPK by phosphorylating its threonine residue 172 at an α catalytic subunit.^{143,}
¹⁴⁴ Previous studies have proved that nobiletin and HPMFs decreases triglyceride content by increasing the phosphorylated AMPK α protein level.^{12, 145} We found that 5-Ac-Nob increased phosphorylated AMPK α and its upstream phosphorylated LKB1 protein levels (Figure 31) The results above showed that 5-OH-Tan, 5-OH-Nob, 5-Ac-Tan, and 5-Ac-Nob decreased triacylglycerol content and did not affect the cell cycle arrest stage during cell differentiation and 5-Ac-Nob could decrease most the triacylglycerol content among these studied compounds. And the anti-adipogenesis mechanism of 5-Ac-Nob was increased phosphorylated LKB1 and AMPK α protein levels and decreased SREBP-1 and FAS protein levels in the 3T3-L1 preadipocyte model (Figure 35).

According to the anti-adipogenesis ability of 5-Ac-Nob in 3T3-L1 preadipocyte model, we further investigated the effect of 5-Ac-Nob in an HFD-induced obesity mice model. Previous studies have shown that 100 mg/kg of nobiletin reduced body weight, WAT

weight, and plasma TG in an HFD-induced animal model.¹²¹ In the current study, we found that mice fed with HFD diet and 25 (LAN group) or 50 mg/kg (HAN group) of 5-Ac-Nob had lower body weight, WAT weight, plasma TG and total cholesterol compared with the HFD group that mice only fed with HFD diet (Figure 32 and Table 8 & 10). The liver is a major organ in glucose and lipid metabolism and is involved in the control of energy balance and body weight.^{146, 147} In severe obesity, the adipocytes are unable to store excess lipid and lipids accumulate in the liver, skeletal muscle, and heart, and the fat around organs may impair their function. Moreover, adipocytes lose their function, increase lipolysis, increase plasma free fatty acid, and increase TG synthesis in the liver.^{3, 42} HPMFs has been proved that it can decrease fat droplets in the liver.¹⁰ In the current study, we found that 50 mg/kg of 5-Ac-Nob significantly decreased liver TG levels (Figure 34.) and its decreasing lipid accumulation mechanism was by increased phosphorylated LKB1 and AMPK α protein levels and decreased phosphorylated ACC protein levels compare with the HFD group in the liver (Figure 35). Determined the level of Glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) is a basic procedure to diagnosis and monitor liver disease and could be further information of the severity of the liver disease.¹⁴⁸ We found the GOT and GPT level in the plasma were in normal range (Table 9 & 10) and the results of liver pathology did not

show any toxicity in the liver (Table 11). All these results show that 5-Ac-Nob decreased body weight and WAT fat, ameliorated hyperdelipidemia, and it had potential to prevent fatty liver by increased the phosphorylation of LKB1 and AMPK α , and affected phosphorylated ACC protein levels to decrease lipid accumulation in liver in the HFD-induced obesity animal model.

In the hypothesis, we assumed that acetylation nobiletin and tangeretin could be a prodrug to make their parent compounds had more efficacies in the body. According to the results mention above 5-Ac-Nob do had better anti-adipogenesis ability than their parent compounds. Therefore, we determine nobiletin, 5-OH-Nob and 5-Ac-Nob level in plasma within 120 minutes after the mouse given 100 mg/kg 5-OH-Nob or 5-Ac-Nob try to understand their adsorption in plasma In this study, our results suggest that 5-OH-Nob potentially could be a metabolite from oral administration of 5-Ac-Nob and lower concentration of 5-Ac-Nob in plasma. Take aspirin as (acetylsalicylic acid) an example, previous studies have indicated that many factors affected the rate of absorption of aspirin including the pH and surface area of the gastrointestinal, the rate of gastric emptying. Besides, different sites of blood vessels also showed different blood concentrations of aspirin during the absorption phase. During metabolism of aspirin *in vivo* it was partially bound to plasma protein and hydrolyzed to salicylate.^{13, 149} Since all these factors could

affect the concentrations of aspirin in plasma might be lower than the limit of quantification due to incomplete bioavailability and hydrolysis to salicylate during the absorption phase^{13, 150} In our study, 5-Ac-Nob could be hydrolyzed to 5-OH-Nob or acetylated with other proteins that made it has lower concentration in plasma. It needs further experiments to evaluate the absorption, distribution, bioavailability and biotransformation of 5-OH-Nob and 5-Ac-Nob to clarify how 5-Ac-Nob works *in vivo* and *in vitro*.

CHAPTER 8. CONCLUSION

According to the data provided in previous chapters, 5-Ac-Nob is the best candidate for anti-adipogenesis activity. The major anti-adipogenesis mechanism of 5-Ac-Nob occurs in the late stage of cell differentiation through decreased FAS protein levels and downregulated SREBP1 transcriptional factor and activated phosphorylation of LKB1 and AMPK α to decrease lipid accumulation in 3T3-L1 preadipocyte model. 5-Ac-Nob decreased body weight, intra-abdominal fat WAT weight (perigonadal fat, retroperitoneal fat, and mesenteric fat), plasma TG, and total cholesterol in an HFD-induced obesity animal model. 5-Ac-Nob also had potential to prevent fatty liver by reduced liver TG level through activated-phosphorylated LKB1 and AMPK α protein expression and

inactivated ACC protein expression in liver. 5-Ac-Nob did not cause toxicity, and AMPK α plays an important role in regulating lipid accumulation *in vitro* and *in vivo* (Figure 42). We first illustrated that 5-OH-Nob could be a metabolite from oral administration of 5-Ac-Nob. Overall, 5-Ac-Nob could be a candidate for preventing obesity or obesity-related liver disease without toxicity.

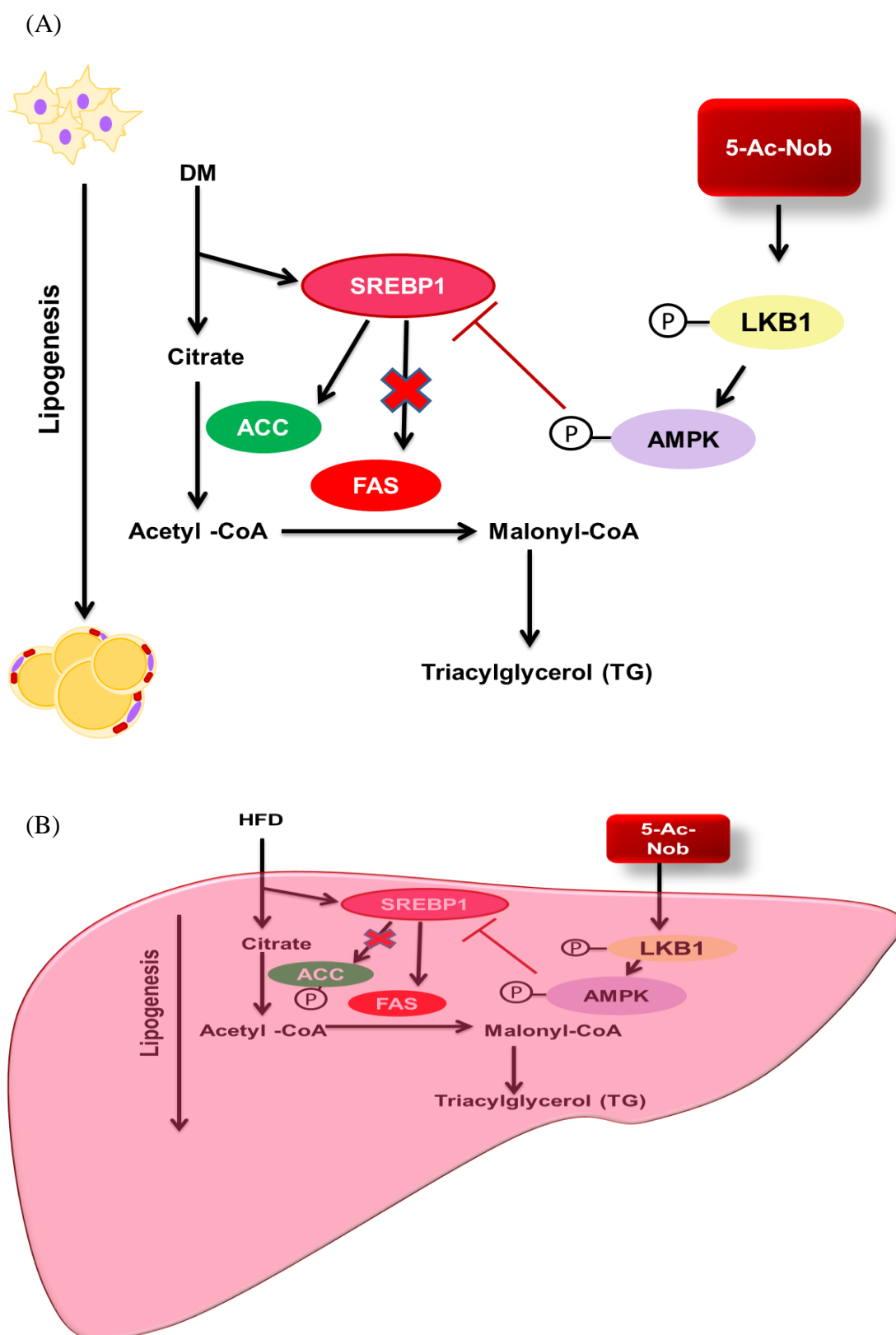


Figure 42. Schematic representation of anti-adipogenesis mechanism of 5-Ac-Nob in (A) a 3T3-L1 preadipocyt model and (B) the liver (HFD-induced obesity animal model)

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