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Glucuronidated flavonoids in neurological protection: structural analysis and approaches for chemical and biological synthesis

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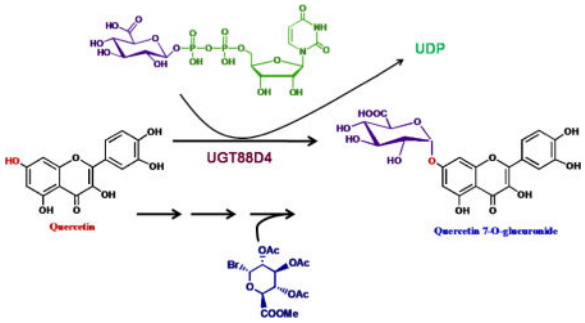
Abstract

Both plant and mammalian cells express glucuronosyltransferases that catalyze glucuronidation of polyphenols such as flavonoids and other small molecules. Oral administration of select polyphenolic compounds leads to the accumulation of the corresponding glucuronidated metabolites at μM and sub-μM concentrations in the brain, associated with amelioration of a range of neurological symptoms. Determining the mechanisms whereby botanical extracts impact cognitive wellbeing and psychological resiliency will require investigation of the modes of action of the brain-targeted metabolites. Unfortunately, many of these compounds are not commercially available. This article describes the latest approaches for the analysis and synthesis of glucuronidated flavonoids. Synthetic schemes include both standard organic synthesis, semi-synthesis, enzymatic synthesis and use of synthetic biology utilizing heterologous enzymes in microbial platform organisms.

Graphical abstract



Keywords

biosynthesis; flavonoid; glucuronide; neurological disorder; organic synthesis; synthetic biology

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■ INTRODUCTION

Flavonoids represent one of the major classes of plant specialized metabolites, and are synthesized through the phenypropanoid/polymalonate pathways, with the aromatic B-ring being derived from L-phenylalanine and the aromatic A-ring derived from condensation of three molecules of malonyl CoA by the plant polyketide synthase known as chalcone synthase[1]. Enzymatic isomerization of chalcone to flavanone yields the first flavonoid with the characteristic central heterocyclic ring. All other classes of flavonoid are formed biosynthetically in plants through oxidation and reduction reaction reactions occurring on the central C-ring, and diversity within the various classes occurs through various types of modifications to the aromatic A and B rings and the C-ring 3-hydroxyl group[2]. Glycosylation is perhaps the most common of such modifications, but plants also contain families of enzymes capable of catalyzing hydroxylation, *O*-methylation, sulfation, acetylation, prenylation, and other modifications of the flavonoid nucleus[2].

Glycosylated flavonoids are widespread in plants; in fact, sugar substitution of the flavonoid aglycone is generally a prerequisite for transport to and storage of the flavonoid in the central vacuole of the plant cell[3]. Flavonoids can be substituted by a single sugar residue on one hydroxyl group, by a group of linked sugars attached to a single hydroxyl group, or by substitution with two or more sugars at more than one position. In addition, *C*-glycosyl flavonoids occur in which a non-hydrolysable carbon-carbon bond links the sugar directly, usually to an A-ring carbon[4]. Within this broad diversity, sugar substitution at a single position is probably the most common, with glucose as the most prevalent sugar. However, glucuronic acid is also attached to plant-derived flavonoids, and whether a particular flavonoid is glucuronidated or glucosylated in a particular plant tissue will depend on the tissue-specific expression patterns of the plant's suite of glycosyltransferase enzymes and their sugar donor and acceptor specificities. For example, whereas the major flavonoids found in the aerial parts of *Medicago* species are glucuronides of the flavones tricin, apigenin, chrysoeriol and luteolin[5], the roots contain isoflavone derivatives, primarily glucosides and malonylated glucosides of formononetin and medicarpin[6]. However, ectopic expression of the gene encoding the entry point enzyme of isoflavone biosynthesis in transgenic alfalfa (*Medicago sativa*) results in accumulation of isoflavone glucosides, not glucuronides, in the leaves[7].

Flavonoid glucuronides have been ascribed health-promoting activities. Examples include biacalein-7-*O*-β-glucuronide (wound healing promotion and anticancer activity)[8,9], (3-*O*-methyl) quercetin-3-*O*-β-glucuronide (anti-inflammatory and neuroprotective activities)[10–12], 3-methoxyflavonol-4′-*O*-glucuronides (anti-allergenic)[13] and epicatechin glucuronide (promotion of vascular function)[14]. In some of the above cases, the glucuronidation of the flavonoid is the result of mammalian metabolism. Ingestion of flavonoids by animals generally results in the hydrolysis of pre-existing *O*-glycosidic bonds in the digestive system, with further metabolism of the aglycone through the pathways common for metabolism of endo- and xenobiotics, namely phase I modification, phase II conjugation and phase III elimination. Phase II conjugation of flavonoids in mammals commonly involves glucuronidation to generate metabolites that can diffuse into portal and lymphatic circulation[15].

Glucuronidation significantly impacts the physiological properties of the flavonoid such as its solubility (increased), bioactivity (decreased or in some case increased), bioavailability (usually increased), and inter- and intra-cellular transport as well as excretion (usually increased). Not only does the conjugation of flavonoid compounds contribute to their uptake, but the position of glucuronidation also impacts the anti-oxidant and pro-oxidant properties of flavonoids[16,17]; for example, glucosides and the 3-*O*-glucuronide of the stilbene resveratrol exhibit stronger antioxidant activity than trans-resveratrol itself[18].

Recent evidence suggests that glucuronidation and other types of metabolism of flavonoids in animals might, in addition to allowing for secretion, also target bioactive molecules to their sites of action. For example, previous studies by our research group have demonstrated that oral administration of a botanical supplement mixture from grapevine is effective in protecting against neuropathology and cognitive impairment in aging[19,20]. These studies identified 18 biologically available phenolic metabolites, including 16 polyphenol metabolites[21] and two phenolic acids[22] that are found to accumulate in the brain with the potential to protect against Alzheimer's disease pathogenic mechanisms. Moreover, in ongoing studies, we have demonstrated that some of these brain-accumulating polyphenol metabolites, in particular, 3′-*O*-methyl-epicatechin-5-glucuronide[19], quercetin-glucuronide[12], as well as 3-hydroxybenzoic acid and 3-(3′-hydroxyphenyl) propionic acid[22] are capable of contributing to the efficacy of these botanical supplements to interfere with the mechanisms associated with cognitive and psychological resilience.

We are presently characterizing the cellular/molecular mechanisms through which individual flavonoids may contribute to the efficacy of the botanical mixture to modulate, respectively, psychology and cognitive resilience. For example, our studies support the evidence that select phenolic metabolites can contribute to the efficacy of the botanical mixture to promote cognitive resilience by modulating neuronal synaptic plasticity (e.g., by the polyphenol metabolites 3′-*O*-methyl-epicatechin-5-glucuronide and quercetin-glucuronide) as well as c-Fos, Arc, and Erg cellular signaling pathways (e.g., by the phenolic acids homovanillic acid and 3,4-dihydroxyphenylacetic acid resulting from flavonoid catabolism).

Figure 1 shows the basic structures of three of the major classes of flavonoids found in grape seeds and juice; after ingestion, these compounds appear in the brain as glucuronidated and methylated derivatives. To further pursue the potential mechanisms of action of such glucuronidated flavonoids in animal systems, it is necessary to have authenticated standard compounds. However, several of the phase II derivatives of the compounds shown in Figure 1 are not commercially available, including 3′-*O*-methyl quercetin 3-*O*-glucuronide, the 5-*O*-glucuronides of (epi) catechin and 3′-*O*-methyl (epi)catechin, and the 3-*O*-glucuronides of cyanidin, delphinidin and malvidin. Also unavailable are positional isomers (e.g. with the glucuronide or other substituents located on different positions) necessary for a full understanding of structure-activity relationships and identification of target receptor sites. Plants have preferences for glycosylation that also preclude access to some of these compounds from plant sources; for example, anthocyanidins are generally glucosylated, not glucuronidated, at the 3-*O*-position.

Although there is limited understanding of polyphenol metabolism in mammals, we have previously demonstrated that oral administration of certain brain-bioavailable phenolic glucosides, such as malvidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3-glucoside and peonidin-3-glucoside, as well as resveratrol, result in their intact delivery to the brain[21]. However, there is no mechanistic understanding of how specific flavonoid modifications (e.g glucuronidatation) may influence delivery of targeted metabolites to the brain.

Because we are aware that brain concentrations of flavonoid metabolites are too low to allow for extraction and purification in the multi-mg amounts necessary for mechanistic studies, production of flavonoid glucuronides must therefore rely either on chemical synthesis, or biochemical approaches using enzymes, either in vitro or through synthetic biology approaches in host organisms. These approaches, as well as the analytical tools necessary to ascribe structure to biologically modified flavonoids, are outlined and evaluated in the present review.

■ ANALYSIS OF GLUCURONIDATED FLAVONOIDS

The most common methods for the detection and quantification of flavonoid glucuronides in complex matrices are high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS), and the preferred methods for structural characterization are nuclear magnetic resonance (NMR) spectroscopy, tandem mass spectrometry (MS/MS), and X-ray crystallography.

A drawback of both NMR and X-ray crystallography is the requirement for quite large amounts of purified compounds. This problem is partially alleviated by the use of hyphenated mass spectrometry techniques, including continuous-flow fast-atom bombardment (CF-FAB)[23–25], MALDI-ToF[26,26–29], and electrospray (ES)[23,30–33], which allow for elucidation of flavonoid glucuronide structures based on accurate molecular weights and diagnostic fragmentation patterns. Capillary electrophoresis[23,34,35], HPLC or UPLC[36–38] and, latterly, HPLC–MS[39–47] are now routine and provide advanced separation methods that have facilitated the solution of previously intractable flavonoid structures. Although gas chromatography (GC) coupled to MS can been used for glycosylated flavonoid analysis, it is not widely applicable in the present case because glucuronides exhibit limited volatility, necessitating time-consuming derivatization; the fragmentation patterns of these derivatives are also often hard to interpret.

Mass spectrometry

MS is the preferred detection technique for analysis of flavonoid glucuronides[48–59], primarily because it only requires very small quantities of analyte to generate accurate tandem mass (MS/MS) spectra. Characteristic molecular ions are formed; either protonated $[M + H]^+$ or ammonium or alkali-metal ion adducts in the positive-ion full-scan mode, or the $[M - H]^-$ ion in the negative-ion mode[60].

In the positive ion mode, the ions that are formed from cleavage of two bonds in the C-ring are denoted as $^{i,j}A^+$ and $^{i,j}B^+$, with ion A comprising the A-ring and ion B the B-ring[61]. The C-ring bonds that are broken are represented by the indices i and j. These ions are denoted as

$^{i,j}A^-$ and $^{i,j}B^-$, respectively, when using the negative ion mode. For glucuronidated flavonoids, it is important to avoid confusion with the Ai^+ and Bi^+ ($i \geq 1$) labels that designate fragments containing a terminal (non-reducing) glucuronic unit (Figure 2)[57,6]; for this purpose, an additional subscript 0 is used to the right of the letter.

A diagnostic fragment of $[M+H-176]^+$ is commonly observed on analysis of glucuronides carried out in the positive-ion mode. Because the negative charge is retained on the glucuronide moiety, an abundant glucuronate fragment (*m/z* 193) is often seen in the negative-ion mode. Subsequent dehydration, yielding a less abundant ion at *m/z* 175, followed by successive losses of CO2 and H2O (*m/z* 113) and CO (*m/z* 85) (Figure 3), are also seen[62–64].

Absolute structural characterization of the sites of conjugation of positional isomers of flavonoid glucuronides is always difficult by MS. However, flavonoids are good chelating agents towards metal ions, and this has led to novel approaches for differentiating positional isomers by the formation of metal adducts with characteristic fragmentation[65–67]. The favored sites of chelation by iron, cobalt and copper are catechol groups, hydroxyl groups adjacent to oxo groups, and 1-oxo-3-hydroxyl-containing moieties[68]. This approach enhances the capabilities of MS[69–71], allowing isomeric metabolites to be differentiated under CID conditions.

Nuclear magnetic resonance spectroscopy

Although NMR spectroscopy is a powerful technique for determining the structures of flavonoid glucuronides[72–78], it is limited by poor sensitivity, low throughput, and difficulties resolving components in mixtures. It is, however, possible to completely assign all proton and carbon signals for most flavonoids using a few mg of sample[79–82], based on chemical shifts (δ) and spin-spin couplings (coupling constants (*J*)) with comparison with compiled data. In addition to identifying the type of aglycone and substituent groups, NMR analysis also identifies the number and anomeric configurations of the attached glucuronide moieties. ^{1}H NMR data can be complemented with results from ^{13}C NMR experiments; however, ^{13}C NMR is much less sensitive due to the low abundance of ^{13}C (1.1%) compared to ^{1}H (99.9%)[83].

Unequivocal structural elucidation of flavonoid glucuronides by NMR requires various 2-D approaches. These yield contour maps showing the correlations between different nuclei in the molecules, either between the same (homonuclear) or diffferent (heteronuclear) elements[83]. Homonuclear ^{1}H–^{1}H correlated NMR techniques include double-quantum filtered COSY (^{1}H–^{1}H DQF-COSY), ^{1}H–^{1}H TOCSY, ^{1}H–^{1}H NOESY and rotating frame Overhaüser effect spectroscopy (^{1}H–^{1}H ROESY). Homonuclear experiments generate spectra in which ^{1}H chemical shifts are correlated with each other along two axes. In contrast, heteronuclear NMR experiments such as ^{1}H–^{13}C HSQC and heteronuclear multiple bond correlation (^{1}H–^{13}C HMBC) show ^{1}H–^{13}C correlations as crosspeaks in the spectrua[83–85].

A comparison of the chemical shifts of the glucuronide to those of the aglycone can reveal the site of glucuronidation, with the largest changes in chemical shifts in the glucuronide

being found in the atoms near the site of conjugation[79,80]. A recent study on the isolation and characterization of several new flavonol glucuronides from the flower buds of *Syzygium aromaticum* (clove) well illustrates the use of combined NMR and MS approaches[79]. The compounds characterized were rhamnetin-3-*O*-β-D-glucuronide **(1)**, rhamnazin-3-*O*-β-D-glucuronide **(2)**, rhamnazin-3-*O*-β-D-glucuronide-6″-methyl ester **(3)**, and rhamnocitrin-3-*O*-β-D-glucuronide-6″-methyl ester **(4)**. As an example, Figure 4 shows HMBC correlations for compound 1 reported in[79].

High-Performance Liquid Chromatography–Nuclear Magnetic Resonance Spectroscopy

Although LC–UV–MS and LC–MS–MS can often provide sufficient information to enable the identification flavonoids and their glycosides, additional analytical power is provided by the combined approach of LC–NMR (Figure 5). Generally, LC–UV–MS and LC–UV–NMR are run separately. Their coupling provides a very powerful approach in which the separation and structural elucidation of unknown compounds in even quite complex mixtures are combined[40,86,–91].

Recent progress in pulse field gradients, solvent suppression, probe design, and construction of high-field magnets have significantly improved the technique. ^{1}H NMR spectra are obtained from selected peaks in the HPLC chromatogram, complementing the LC–MS data, from which the nature of substituent groups can be deduced from the fragmentation pattern but their exact positions cannot be determined. For simple flavonoids, such as apigenin, the ^{1}H NMR component alone will reveal the substitution position because of the unique splitting pattern for each possible location of the B-ring hydroxyl group[92].

■ STRATEGIES FOR CHEMICAL SYNTHESIS OF GLUCURONIDATED FLAVONOIDS

Both glycosyl donors of the glucuronic acid type and phenolic acceptors pose problems for synthetic coupling. Construction of the correct regiospecific phenol glucuronidic linkage requires, before conjugation with the glucuronyl donors **5–9** (Figure 6), that the phenolic compounds be converted into appropriately protected precursors.

Protection of hydroxyl groups of flavonoids

To achieve glucuronidation of flavonoids, partial or complete protection of the hydroxyl groups is necessary. An approach was developed for selective protection of each hydroxyl group of quercetin **10** by the groups of Rolando[93,94] and He[95] following the scheme in Figure 7. Briefly, the free hydroxyl groups of **10** were benzylated using benzyl bromide and potassium carbonate in DMF at room temperature, leading to a mixture of 3,7,3′,4′-*O*-tetrabenzylquercetin **11** and 3,7,4′-*O*-tribenzylquercetin **12**, which were recovered with 60% and 20% yield, respectively (Figure 7). Alternatively, selective protection of the north-east catechol of quercetin **10** by dichlorodiphenylmethane led to the ketal **13** with 86% yield (Figure 7) to give entry into the series substituted at the 3 position[94,96,97]. Finally, benzylation of quercetin pentaacetate **14** at the 4′ and 7-positions with benzyl chloride/sodium bicarbonate/benzyl(triethyl)ammonium chloride using microwave irradiation (545 W, 160 °C) for 10 minutes gave compound **15**[95].

To allow the regiospecific glucuronidation of a single hydroxyl group of epicatechin with protection of the remaining groups (Figure 8)[98], the specific hydroxyl group to be glucuronidated is protected with a methoxymethyl (MOM) group, while the remaining hydroxyls are protected as benzyl ethers.

Basic Glucoronidation

Glucuronidation of phenols by the Koenig–Knorr method using glycosyl bromide donors is probably the most reliable approach, although yields can be relatively low[99–101]. For synthesis of quercetin glucuronide by this approach, 4′,7-dibenzylquercetin **15**[102] was treated with methyl 2,3,4-tri-*O*-acetyl-1-bromo-α-D-glucuronate **5**/silver oxide (Ag2O) at 0°C to give a 52% yield of glucuronidated product; the final deprotection using Na_2CO_3 in aqueous MeOH was more efficient[103,104]. The overall yield was considerably increased by reaction below room temperature[104]. A similar approach has also been attempted for the glucuronidation of unprotected catechin, but the results are a mixture of glucuronidated catechins[105]. Other bases have been used as catalysts in this reaction such as LiOH, K_2CO_3, Ag_2CO_3, $AgClO_4$, AgOTf, $Hg(CN)_2$ or $CdCO_3$[106,107]. A by-product, a 2-acyloxyglycal **20** (Figure 9) arising from HBr elimination from **5**, has been frequently observed when using the Koenig–Knorr reaction[104,108]. This most likely arises from use of basic catalysts such as Ag2O.

The synthesis of quercetin 3-glucuronide **23** was first reported by Wagner in 1970[109]. Subsequently, Needs and Kroon[104] carried out a selective glucuronidation of **15** with methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl) uronate bromide **5** in the presence of pyridine and Ag_2O at 0°C, using 3 Å molecular sieves to ensure anhydrous conditions. The reaction gave **22a** and **22b** in a combined yield of 52% (Figure 10). A three step debenzylation and ester hydrolysis afforded **23** in 40% overall yield from **15**.

To synthesize malvidin-3-*O*-β-glucuronide via the Koenigs–Knorr reaction, the reaction proceeded via α-hydroxyacetosyringone **25** that was formed in three steps from acetosyringone **24** by the method reported by Luis and Andres[110]. The glucuronidation reaction to form the ‘Eastern part’ **26** employed the Koenigs–Knorr reaction[106] between **25** and bromo-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronic acid methyl ester **5**, refluxing with silver carbonate as base in dry toluene. The reaction products were a mixture of the mono- and di-glucuronic acetophenone derivatives (**26a, 26b**). The C ring was generated by an aldol-type condensation between compounds **27**, Western part, and **26a**, Eastern part, following the deprotection steps to afford malvidin-3-*O*-β-glucuronide **28** (Figure 11)[111].

Acid glucuronidation

Glucuronidation of compounds with phenolic hydroxyl groups often utilizes methyl (2,3,4-tri-*O*-acetyl-D-glucopyranosyl trichloroacetimidate) uronate **6**, with activation of the coupling step by Lewis acids such as $BF_3{\cdot}OEt_2$, TMSOTf or $ZnCl_2$[99,102,103,112–115]. Use of benzyl uronate counterpart **8** should facilitate the final release of the carboxylic acid function following hydrogenolysis under neutral conditions[116]. This acid glucuronidation reaction generally requires full or partial protection of the phenolic hydroxyls[104,116]. Glycosyl trifluoroacetimidates **7, 9** are valuable alternatives to the corresponding

trichloroacetimidates **6, 8**[114,116–119] and have shown advantages in synthesis of flavanone glucuronides[120].

For synthesis of quercetin 3′-*O*-glucuronide under selective acid glucuronidation, treatment of **15** with methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate trichloroacetimidate **6** in the presence of 3 Å molecular sieves and dry CH_2Cl_2 gave **29** and recovered **15** (Figure 12). Glucuronidation at the 3-*O*-position was not observed. Debenzylation and de-esterification afforded, after purification, **30** in 11% yield. It is important to note the differences of regioselectivity between the reactions with glucuronyl donors **5** and **6**[104].

For the chemical synthesis of epicatechin glucuronides, compounds **18a** and **18b** are suitably protected for *O*-glucuronidation specifically at positions 3′ and 4′, respectively. This was achieved using the glucuronic acid donor **7**, under BF_3•OEt_2 catalysis. Mild alkaline hydrolysis, followed by hydrogenolysis over $Pd(OH)_2$/C, yielded the glucuronides **32a** and **32b** (Figure 13)[113,121].

Regio- and stereo-selective synthesis of quercetin *O*-β-D-glucuronidated derivatives via selective and non-selective glucosylation of quercetin

Glucuronidation provides additional challenges when compared with the more usually performed glucosylation of natural products[113]. This is highlighted in the case of polyphenols, for which even glucosylation can be problematic. For example, the yield of quercetin-7-*O*-glucuronide was only 8% following alkylation of 3,3′,4′,5-tetrabenzoylquercetin by glucuronic acid methyl ester bromide triacetate **5**[122]. In contrast, efficient glucosylation of flavonoids occurs under mild conditions using a phase transfer catalyst such as tetrabutylammonium bromide[123]. Synthetic procedures have been developed for the formation of quercetin glucuronides based on the sequential and selective protections of the hydroxyl functions to allow selective glucosylation, followed by TEMPO-mediated oxidation of the glucoside to the glucuronide. These technologies make it possible to synthesize the four *O*-β-D-glucuronides of quercetin[93].

The most common glycosidation of quercetin is at position 3 of the C ring. Quercetin 3-*O*-β-D-glucuronide **23** can be synthesized from compound **13** in five steps (Figure 14): (i) selective glucosylation of the 3-hydroxyl group; (ii) protection of the remaining free 5 and 7 hydroxyl groups with benzyl bromide in excess K_2CO_3 in dimethylformamide at room temperature); (iii) deprotection of the sugar residue by removal of the acetoxy group with sodium methylate; (iv) selective oxidation by NaOCl (catalyzed by TEMPO) of the sugar of quercetin-3-*O*-β-D-glucoside **35** with the phenol groups still protected, with solubility of **35** ensured by phase transfer catalysis between CH_2Cl_2 and saturated sodium hydrogencarbonate with tetrabutylammonium; and finally (v) deprotection of the hydroxyl groups of the flavonoid by catalytic hydrogenation using 30% palladium on charcoal to yield the 3-*O*-β-D- glucuronide **23** (25%)[93,94].

It is also possible to carry out the non-selective glycosylation of the A-ring 5-position of quercetin, which is less reactive than the 3-position. In this case, the protocol includes a first protection of the 3, 3′, 4′ and 7 hydroxyl groups, which are not to be glycosylated. The glycosylation is achieved on the 3, 3′, 4′, 7-tetrabenzylated quercetin **11** (Figure 15). The

synthesis of quercetin-5-*O*-β-D-glucuronide **38** proceeds in four steps: (i) tetrabenzylated quercetin **11** is reacted with acetobromoglucose in the presence of potassium carbonate; (ii) the glucoside moiety is deprotected as described above; (iii) oxidation of the primary alcohol of quercetin 5-*O*-β-D-glucoside with protected phenol groups **37** is performed to form the corresponding protected glucuronide. Finally, deprotection by removal of the benzyl groups generates quercetin 5-*O*-β-D-glucuronide **38** with 25% yield[93].

Selective glycosylation of the B ring of quercetin can be performed starting from the tribenzylated quercetin **12**; the coupling reaction is carried out under phase transfer conditions with acetobromoglucose, as discussed earlier (Figure 16). Position 5 is not protected as it is not reactive. Deprotection of the glucoside moiety is performed as described above. Finally, selective oxidation of the primary alcohol and deprotection of the hydroxyl groups results in quercetin 3′-*O*-β-D-glucuronide **30** (24% yield)[93].

■ ENZYMATIC GLUCURONIDATION OF FLAVONOIDS

Plants, animals and microorganisms possess enzymes capable of glycosylating a range of plant-derived flavonoid compounds. These enzymes, members of the uridine diphosphate (UDP)-glycosyltransferase (UGT) superfamily, generally possess a common protein structure as well as a 44 amino acid residue signature sequence (the PSPG box) for binding to the UDP moiety of the UDP-sugar that serves as the sugar donor (UDP-glucuronic acid in the case of the UDP-glucuronosyltransferases)[124]. Regioselectivity (i.e. the position of conjugation of the sugar on the flavonoid) depends on the type of flavonoid and the nature of the enzyme catalyzing the conjugation reaction. For example, glucuronidation of the flavone luteolin and the flavonol quercetin in mammals does not follow the same pattern, with regioselectivity depending on the individual flavonoid and the class of UDP-glucuronosyltransferase isoenzyme involved[16]. Because of this specificity, UGTs provide excellent catalysts for biochemical synthesis of sugar conjugates, using simple reaction conditions that do not require protection of non-reacting hydroxyl groups.

Animal enzymes

Mammals, including humans, have evolved a wide range of UGT enzymes and isozymes for glucuronidating compounds, with varying degrees of catalytic efficiency and promiscuity in terms of substrate preference. In humans, there are 27 *UGT* gene products identified, and these are key phase II drug metabolizing enzymes that play central roles in metabolizing and detoxifying foreign chemicals such as carcinogens and hydrophobic drugs[125]. Mammalian UGT1A1 (expressed in liver), UGT1A8 (intestine), UGT1A9 (liver) and UGT1A3 are highly active in conjugating flavonoids (e.g. quercetin and luteolin), whereas UGT1A4 and UGT1A10 and the isoenzymes from the UGTB family, UGT2B7 and UGT2B15, are less efficient[16,126].

The presence of this wide range of UGTs in mammals can be attributed in part to herbivore (mammal): plant: microbe co-evolution as animal herbivores have had to deal with ingestion of toxic phytoanticipins (pre-formed antimicrobial substances) and phytoalexins (inducible antimicrobial substances produced in plants in response to microbial pathogens)[127]. The relative promiscuity of the enzymes allows a range of mammalian tissues, including

intestines, liver, and kidney, to effectively detoxify phytoalexins, phytoanticipins, and drugs[124].

The sugar acceptor specificities of mammalian UDP-glucuronosyltransferases vary considerably. UGT1A1 is perhaps the most important drug-conjugating and xenobiotic detoxifying UGT because of its broad substrate specificity. Table 1 shows the tissue location, substrate preferences and regiospecificities for flavonoids of the human UDP-glucuronosyltransferases. Because of the differential tissue distributions of enzymes with different substrate- and regio-specificities, different tissues function to detoxify flavonoid compounds in different manners. For example, glucuronidation of prunetin (a methylated derivative of the isoflavone genistein and a potential prodrug for cancer prevention) by liver UGT1A7, UGT1A8, and UGT1A9 yielded prunetin-5-*O*-glucuronide whereas intestinal UGT1A1, UGT1A8, and UGT1A10 produced prunetin-4′-*O*-glucuronide[128].

Although glucuronidation, sulfation, and methylation of compounds such as flavonoids are now well established features of phase II endo- and xeno-biotic metabolism, the underlying mechanisms for flavonoid uptake into portal and lymphatic circulation still require elucidation[129].

One major challenge in using mammalian UDP-glucuronosyltranferases in biotechnology applications for the synthesis of flavonoid glucuronides is that the mammalian enzymes are membrane bound proteins. Heterologous expression of these enzymes for novel applications has therefore often proven difficult. Commercial preparations of these enzymes are usually available as supersomes or microsomes prepared from mammalian sources, or alternatively can be obtained by transfecting cDNAs encoding human UGTs into mammalian or insect cell lines, as first demonstrated more than 25 years ago[130]. Such preparations have been used for the biochemical synthesis of glucuronides of epicatechin, catechin, and their 3′-*O*-methyl esters[131,132]. However, relatively large amounts of enzyme are required. These factors of difficulty, expense, and possibly safety, weigh against the use of mammalian enzymes for the biochemical synthesis of flavonoid glucuronides for therapeutic applications such as in the treatment of neurological disorders. At the same time, the purely chemical approaches to the glucuronidation of these compounds as reviewed above are complex and time-consuming. An alternative approach is necessary, and the UDP-glucuronosyltransferase enzymes from plants provide several advantages for safe, rapid and efficient synthesis of flavonoid glucuronides.

Plant enzymes

Glycosylation of phytochemicals mediated by UGTs is one of the major factors determining plant natural product bioactivity and bioavailability[133,134], and many UGTs have evolved for glycosylating plant natural products. For example, about 107 *UGT* genes have been identified in the genome of *Arabidopsis thaliana*[135] and over 300 *UGT* genes are present in the genome of the model legume *Medicago truncatula*[136]. Although a large proportion of the enzymes in these two species identified to date are glucosyl transferases with different acceptor specificities, both species do make some glucuronidated flavonoids. To date, a small number of UDP-glucuronosyltransferases have been identified and functionally characterized from a few plant species; these include flavonoid 7-*O*-

glucuronosyltransferases (F7GATs that comprise UGT88D1, UGT88D4, UGT88D6 and UGT88D7) from members of the Lamiales 137; BpUGT94B1, which is a glucuronosyltransferase of cyanidin-derived flavonoids from red daisy[138]; UBGAT (UGT88D1), purified from cultured cells of *Scutellaria baicalensis* Georgi, that conjugates the 7-OH of the 5-deoxy flavonoid baicalein with glucuronic acid[139]; and the flavonol-3-*O*-glucuronosyltransferase VvGT5 from grapevine (*Vitis vinifera*)[140].

In contrast to human enzymes that are membrane-bound proteins, all plant UDP-glucuronosyltransferases discovered to date are soluble, thus making them prime candidates for developing novel phytotherapeutic flavonoid glucuronides. More effort should be devoted to extending the repertoire of natural plant UDP-glucuronosyltransferases capable of modifying flavonoids beneficial to human health, as only a small number of plants that accumulate glucuronidated flavonoids have been investigated in this respect to date. The idea of broadening the repertoire of available biocatalysts by structure based engineering of plant UGTs is discussed in the final section of this review.

Microbial enzymes

Glucuronidation is one of the mechanisms through which some microorganisms naturally metabolize phenolic compounds such as flavonoids, probably as a detoxification mechanism to make the compounds more soluble[141]. The extent of microbial conjugation of compounds with sugars is subject to multiple parameters including the pH, medium composition, temperature, and concentration of the substrate[142].

Streptomyces sp. strain M52104 can bring about the transformation of flavanone (naringenin), flavonol (quercetin) and several stilbenoids (*trans*-resveratrol, rhapontigenin, deoxy-rhapontigenin) into their *O*-*β*-D-glucuronide derivatives. The bioconversions were always β-stereospecific, but not completely regioselective[141]. *Beauveria bassiana* ATCC 7159 and *Cunninghamella echinulata* ATCC 9244 respectively convert quercetin and its disaccharide derivative rutin into their respective glucuronide derivatives[143]. Generally, however, microbes require engineering with a specific glycosyltransferase if they are to be used as effective catalysts for bioconversion of flavonoids, as discussed further below.

■ BIOTECHNOLOGICAL APPROACHES TO FLAVONOID GLUCURONIDATION

A number of studies have addressed the potential use of microbial biotransformation to generate flavonoid glycosides from the corresponding aglycone. In most cases, the microorganism has been *Escherichia coli*, and the sugar attached has been glucose, derived from the host's endogenous pools of uridine diphosphate glucose (UDPG). Examples include the formation of glucosides of the flavone luteolin, isoflavones genistein and biochanin A, and flavonols kaempferol and quercetin by *E. coli* expressing UGT71G1 or UGT73C8 from *Medicago truncatula*[144]. Yields of glucuronidated products following feeding of the bacterial cultures with the aglycone were in the range of 10–20 mg/L.

Fewer studies have used this approach for the formation of flavonoid glucuronides. Because of the smaller pool of UDP-glucuronic acid than that of UDP-glucose in *E. coli*, the level of

sugar donor can limit the overall yield of glucuronidated product. To overcome this limitation, expression of heterologous glucuronidation enzymes has recently been coupled with engineering of the host to increase the pool of endogenous UDP-glucuronic acid. The manipulation of UDP-glucuronic acid biosynthesis was in two steps; firstly, the *araA* gene encoding UDP-4-deoxy-4-formamido-L-arabinose formyltransferase/UDP-glucuronic acid C-4″ decarboxylase, an enzyme that consumes UDP-glucuronic acid as substrate for capsular polysaccharide biosynthesis, was knocked out in *E. coli*, and secondly, the *E. coli* UDP-glucose dehydrogenase (*ugd*) gene that produces UDP-glucuronic acid[145] was overexpressed. Finally, the respective flavonoid glucuronosyl transferases were expressed in the modified *E. coli* strain; AmUGT10 from *Antirrhinum majus* for luteolin and VvUGT from *Vitis vinifera* for quercetin[146]. Using this strategy, luteolin-7-*O*-glucuronide and quercetin-3-*O*-glucuronide were biosynthesized to levels as high as 300 mg/L and 687 mg/L respectively[146]. A similar approach has been used to manipulate the endogenous upstream biosynthetic pathway for sugar donor (UDP-glucuronic acid) accumulation in concert with the heterologous expression of downstream UDP-dependent glycosyltransferase (SbUBGAT) isolated from *Scutellaria baicalensis* Georgi, which catalyzes the glucuronidation of baicalein. As a result, about 797 mg L^{-1} of baicalein-7-*O*-glucuronide were biosynthesized in engineered *E. coli*[147].

Systems-based engineering for flavonoid glucuronide biosynthesis has the potential of becoming a powerful approach to making health promoting flavonoid glucuronides on a scale that will support further research and development. However, because the currently identified plant enzymes do not cover the full range of substrate- and regio-specificities necessary for generation of all mammalian-derived flavonoid conjugates, for this strategy to have its broadest utility, it will be necessary to either discover more UGTs with different specificities, or else broaden the existing substrate- and regio- specificities of available UGTs by protein engineering.

■ STRUCTURE-BASED DESIGN OF NOVEL GLYCOSYLTRANSFERASES

Identification and characterization of new UDP-glucuronosyltransferases, followed by protein modelling, design and engineering, are strategies that can be used to facilitate the synthesis of currently investigated or novel flavonoid glucuronides. Understanding the mechanism of catalysis by UDP-glucuronosyltransferases is critical for structure-based protein design, but as yet no complete UDP-glucuronosyltransferase crystal structure has been solved. Progress to date has focused on plant glucosyltransferases, the crystal structures of six of which have been reported, namely *Medicago truncatula* UGT71G1, UGT85H2, and UGT78G1, which were determined in our lab[148–150], and grape (*Vitis vinifera*) *Vv*GT1[151], *Arabidopsis thaliana* UGT72B1[152], and *Clitoria ternatea* UGT78K6[153]. A crystal structure of the C-terminal domain of human UGT2B7 has also been reported[154]. These studies have provided structural bases for understanding the catalytic mechanism(s) and specificity of UGTs, and also provide a framework for beginning to address the specific features of flavonoid glucuronosyltransferases[155].

The first insight into the mechanism of catalysis by a plant UDP-glucuronosyltransferase used a combination of protein modeling and site-directed mutagenesis followed by analysis

of the substrate specificity of wild-type and mutated forms of the enzyme. *Bp*UGT94B1 from red daisy (*Bellis perennis*) is a sugar-sugar/branch forming glucuronosyltransferase that catalyzes glucuronidation of a sugar already attached to a flavonoid such as cyanidin. Modeling and biochemical studies showed that an arginine residue (Arg25) in the N-terminus near the catalytic histidine is crucial for sugar donor specificity for UDP-glucuronic acid (Figure. 17)[156].

A modeling study of an F7GAT, UGT88D7, in combination with mutagenesis, showed that the key residue Arg350, which would form interactions with the anionic carboxylate of the glucuronic acid moiety of UDP-glucuronic acid, is crucial for defining the sugar donor-specificity of the enzyme for UDP-glucuronic acid[137]. Arginine-140 was shown to be the determinant for UDP-glucuronic acid specificity of grapevine Vv GT5. However, this amino acid residue did not corespond to any of the previously identified amino acid residues necessary for UDP-glucuronic acid specificity, suggesting independent convergent evolution of plant UDP-glucuronosyltranferases across plant species[140].

Structure-based protein engineering has emerged as an attractive approach to manipulate biosynthetic enzymes to generate novel biocatalysts. Structure-based mutagenesis studies on UGTs have shown that it is possible to manipulate both the regioselectivity of glycosylation and the rate of substrate turnover by site-directed mutations. For example, the F148V and Y202A mutants of UGT71G1 glycosylated quercetin at the 3-*O*-position, compared to the wild-type enzyme that predominantly acts on the 3′-*O*-position[149]. The I305T mutation of UGT85H2 enhanced enzyme catalytic efficiency 37-or 19-fold with kaempferol or biochanin A as sugar acceptors, respectively[148].

Structure-based modifications of plant UDP-glucuronosyltransferases to alter pocket topology, size and composition, presents a new approach for the design of biocatalysts for synthesizing a range of bioactive glucuronides for basic studies and treatment of metabolic syndrome, Alzheimer's disease, and other neurological disorders.

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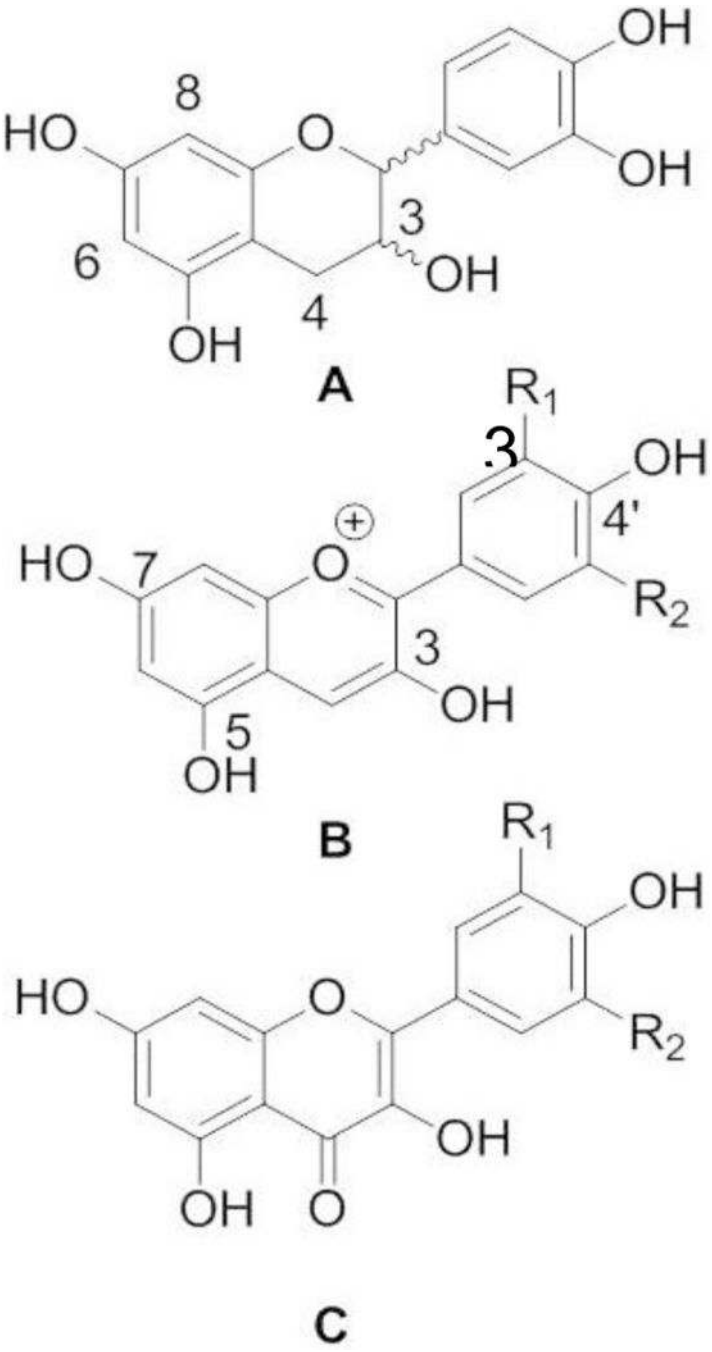

Figure 1.

Structures and numbering convention of three classes of flavonoids that undergo glucuronidation in mammalian tissues. **A**, flavan-3-ol; the stereochemistry of the aromatic ring and 3-hydroxyl denoted with ~~~ determines the catechin (2,3-*trans*) and epicatechin (2,3-*cis*) series. **B**, anthocyanidin (R_1 =OH, R_2=H = cyanidin; R_1=R_2=OMe = malvidin). **C**, flavonol, R_1= OH, R_2 = H = quercetin). The glucuronic acid is commonly attached via linkage to the 5-OH group in the flavan-3-ols, but is usually attached to the 3-OH in anthocyanidins and flavonols.

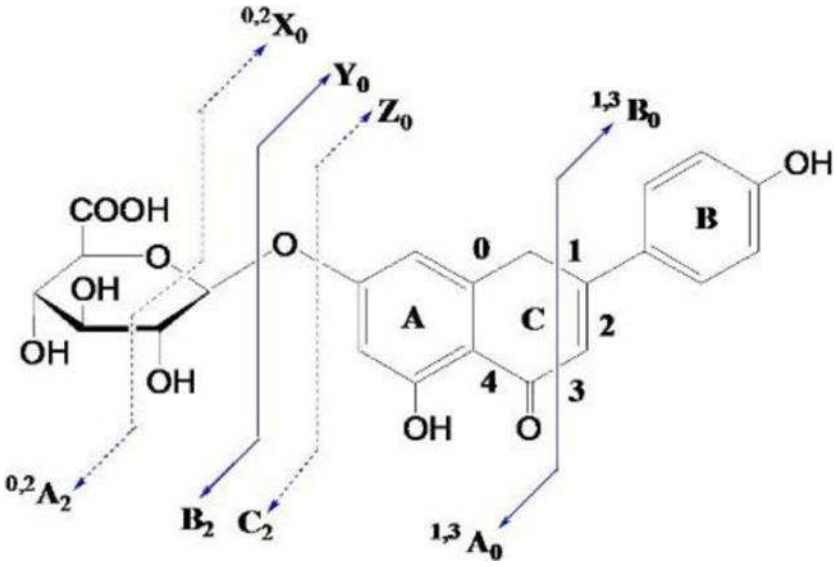

Figure 2.

Ion nomenclature used for flavonoid glucuronides (adapted from[57]). $^{1,3}A_0$ and $^{1,3}B_0$ refer to aglycone fragments containing A- and B-rings, respectively, and superscripts 1 and 3 indicate the broken C-ring bonds. Ai, Bi, and Ci refer to fragments containing glucuronide fragments, with charges retained on the carbohydrate moiety, where i represents the number of broken glucuronidic bonds, counted from the terminal sugar. X_j, Y_j, and Z_j refer to ions containing the aglycone and j is the number of the interglycosidic bond cleaved, counted from the aglycone.

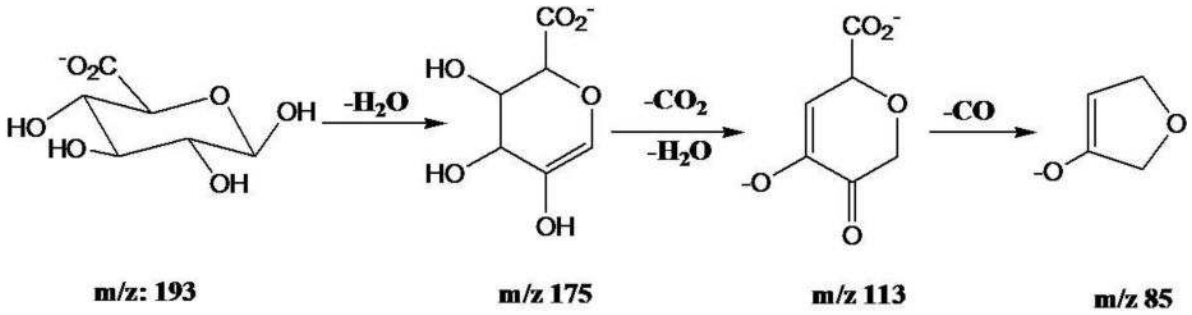

Figure 3.
Characteristic fragmentation of glucuronides in negative MS/MS spectra (adapted from[63]).

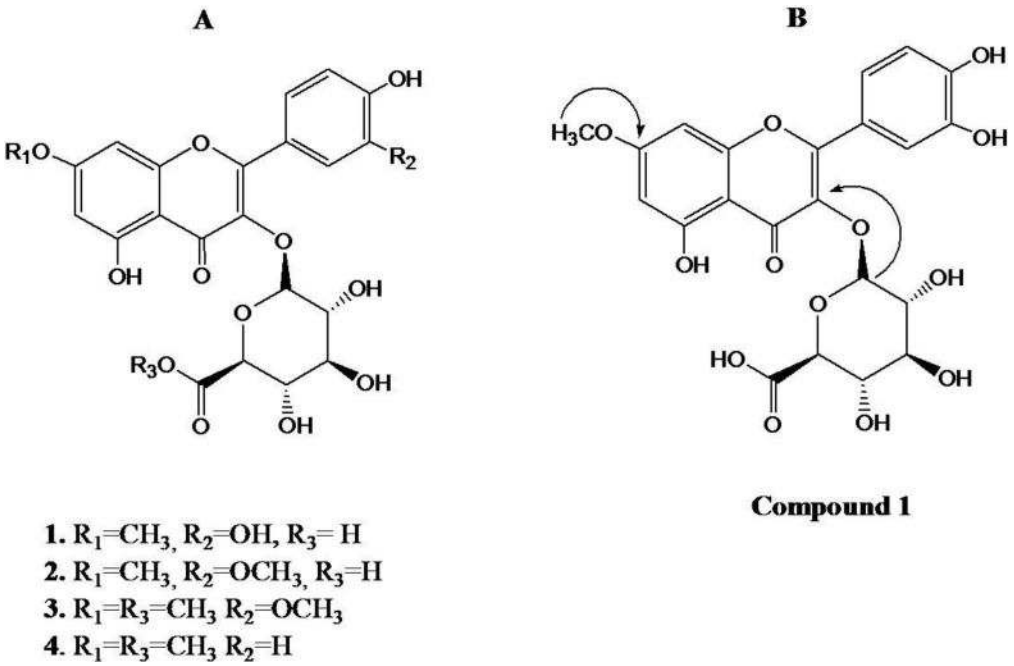

Figure 4.

A, Chemical structure of compounds 1–4 from Jang *et al.* (*79*). **B**, HMBC correlations of compound 1.

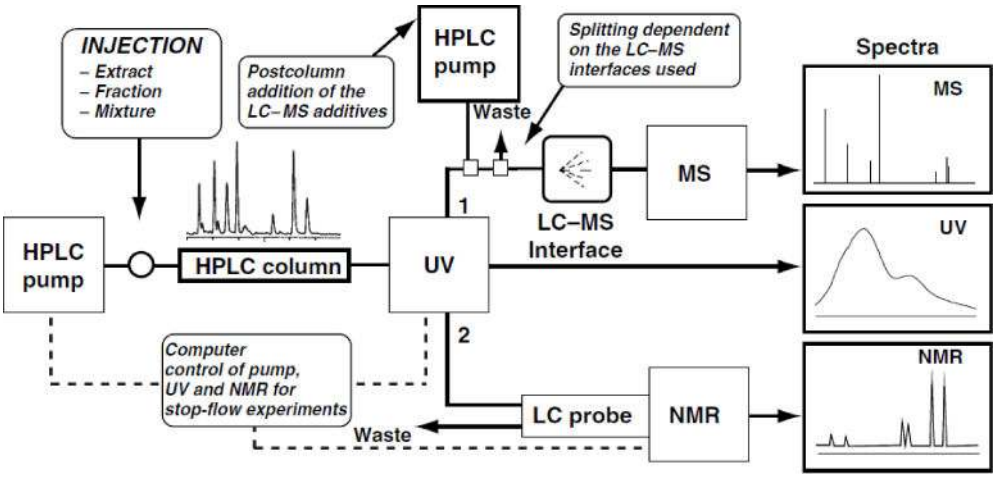

Figure 5.

Schematic representation of the instrumentation used for LC–UV–MS (1) and LC–UV–NMR (2) analyses (adapted from[64]).

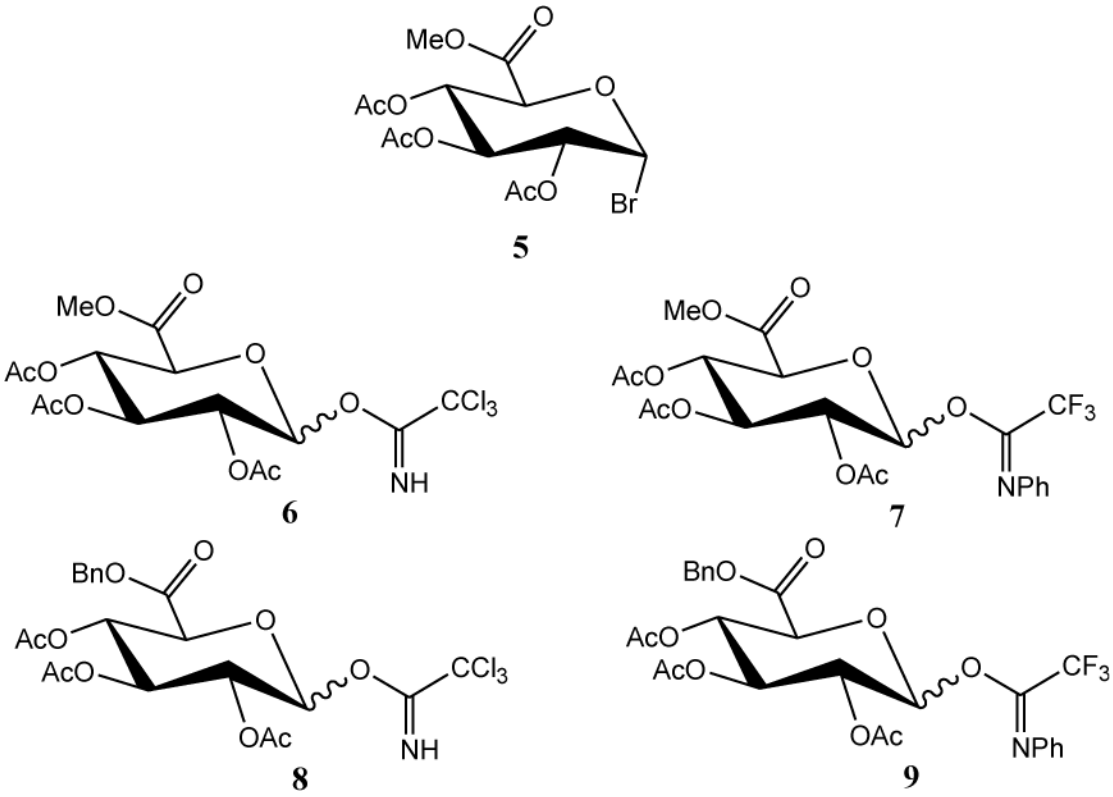

Figure 6.
Glucoronyl donors **5**–**9**

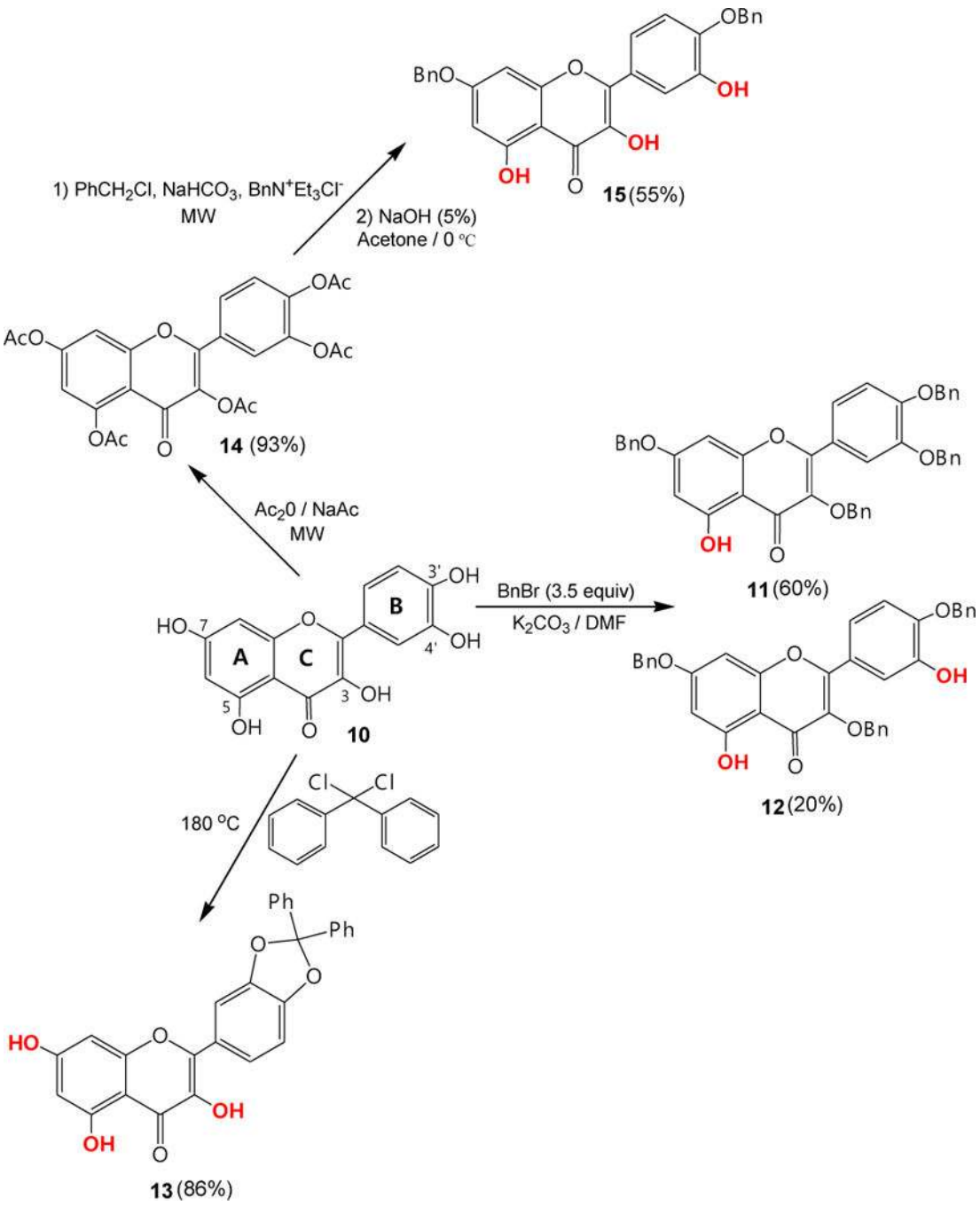

Figure 7.
Selective protection of hydroxyl groups of quercetin **10**.

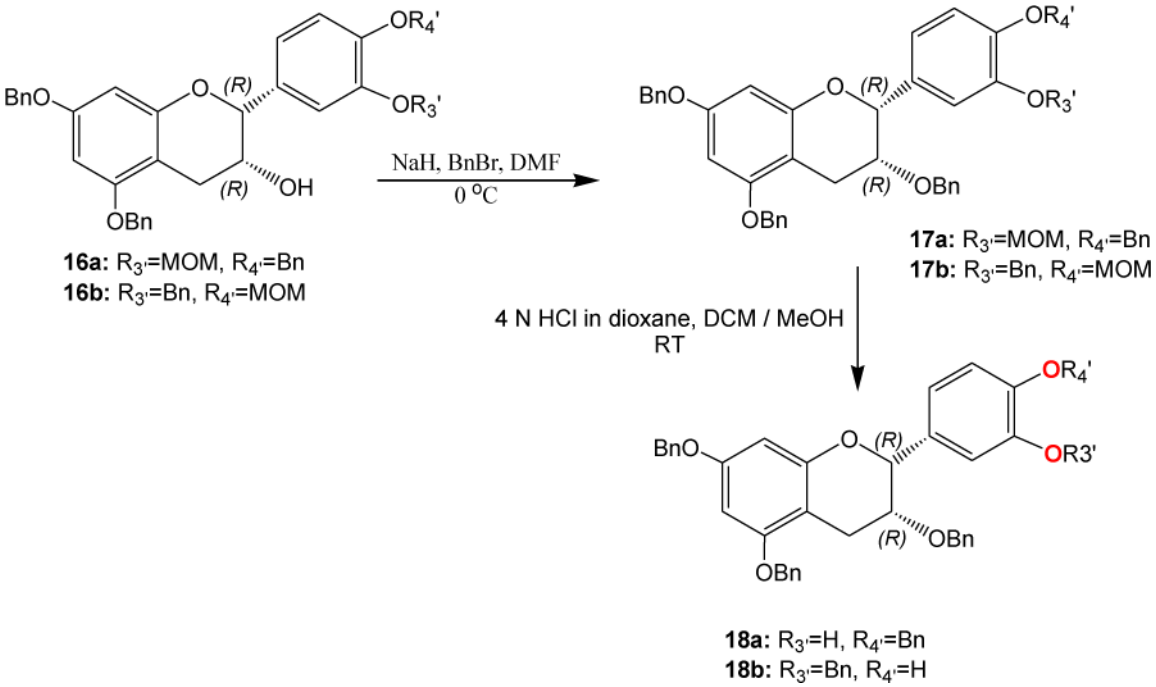

Figure 8.
Synthesis of the protected (*R, R*)-epicatechin derivatives **18**.

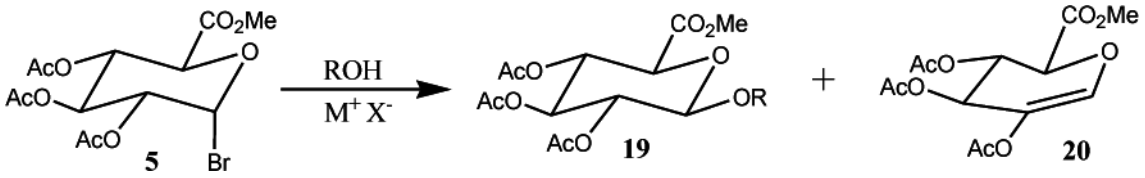

Figure 9.
The elimination by-product (2-acyloxyglycal, **20**) from the Koenig–Knorr reaction.

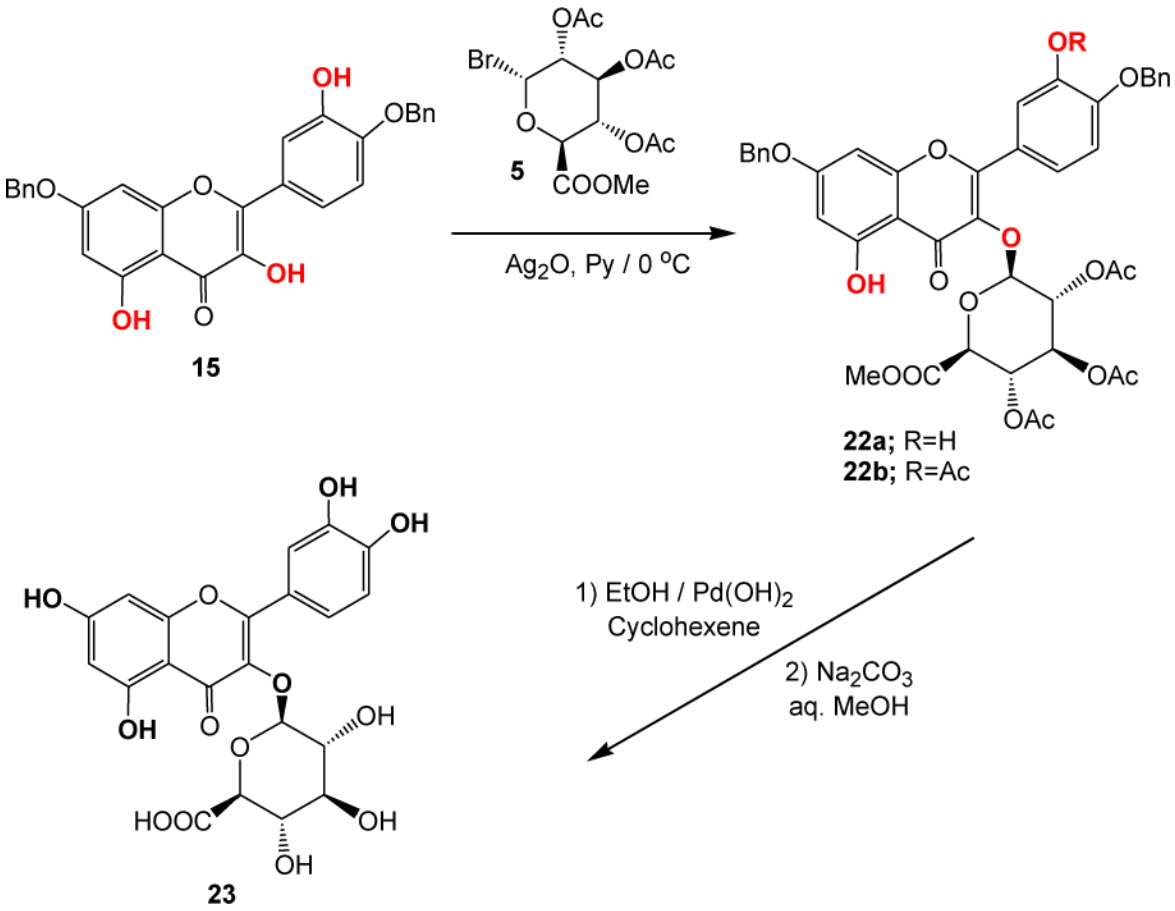

Figure 10.
Synthesis of quercetin-3-*O*-β-D-glucuronide **23** under basic glucuronidation.

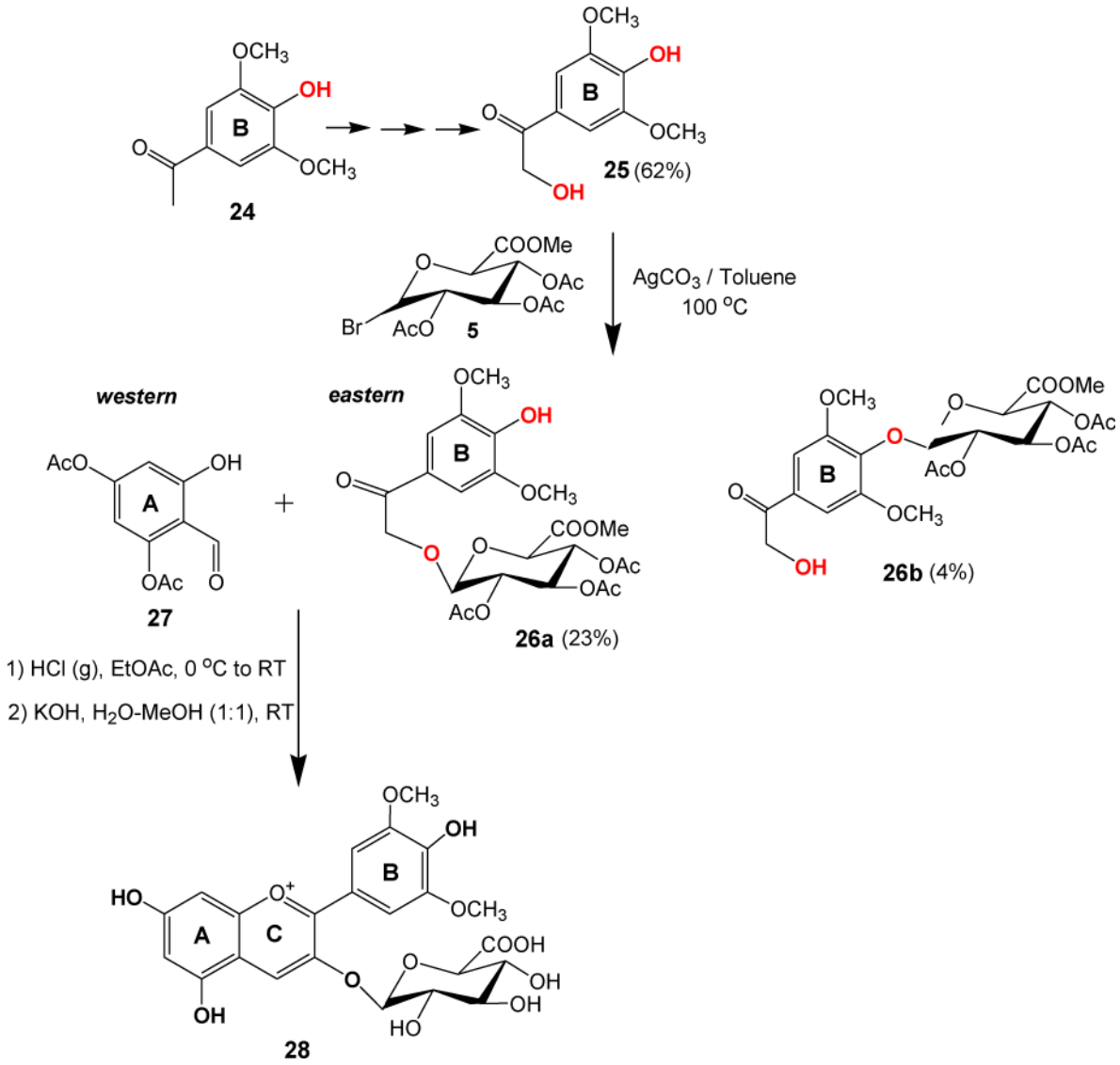

Figure 11.
Synthesis strategy to obtain malvidin 3-*O*-β-glucuronide **28**.

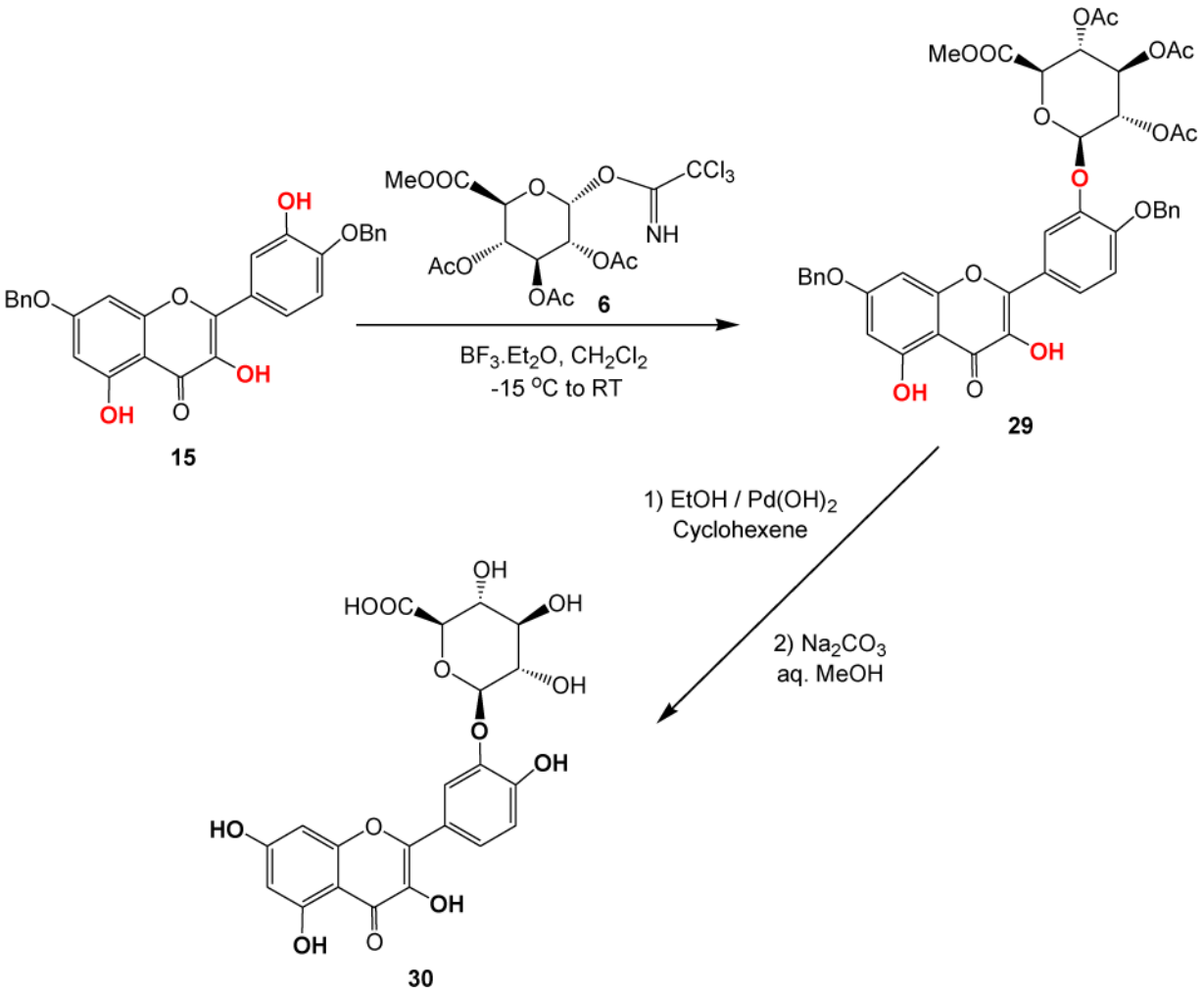

Figure 12.
Synthetic route to obtain quercetin 3′-*O*-glucuronide **30**

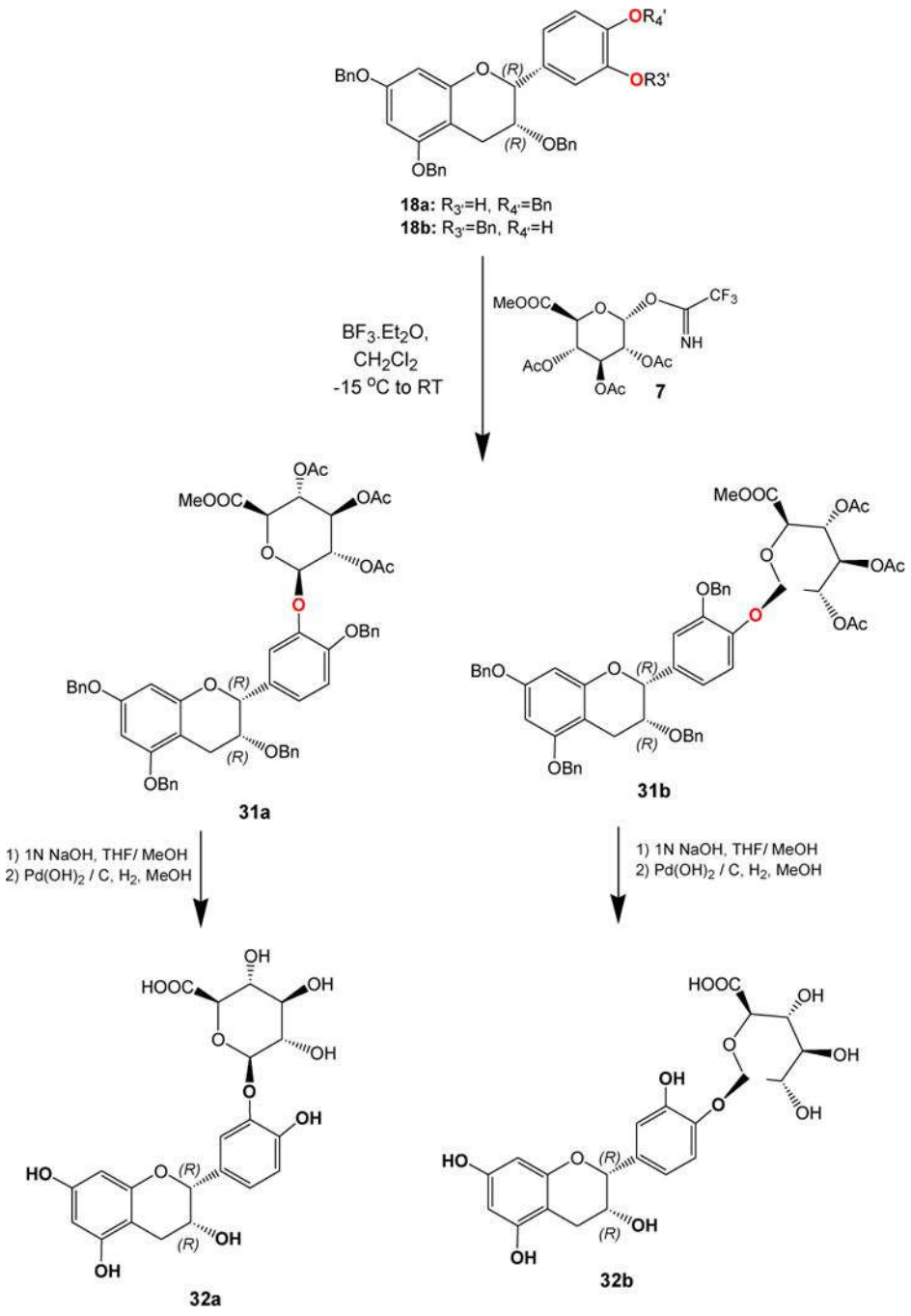

Figure 13.
Syntheses of (−)-epicatechin 3′- and 4′-*O*-β-D-glucuronides **32a–b**.

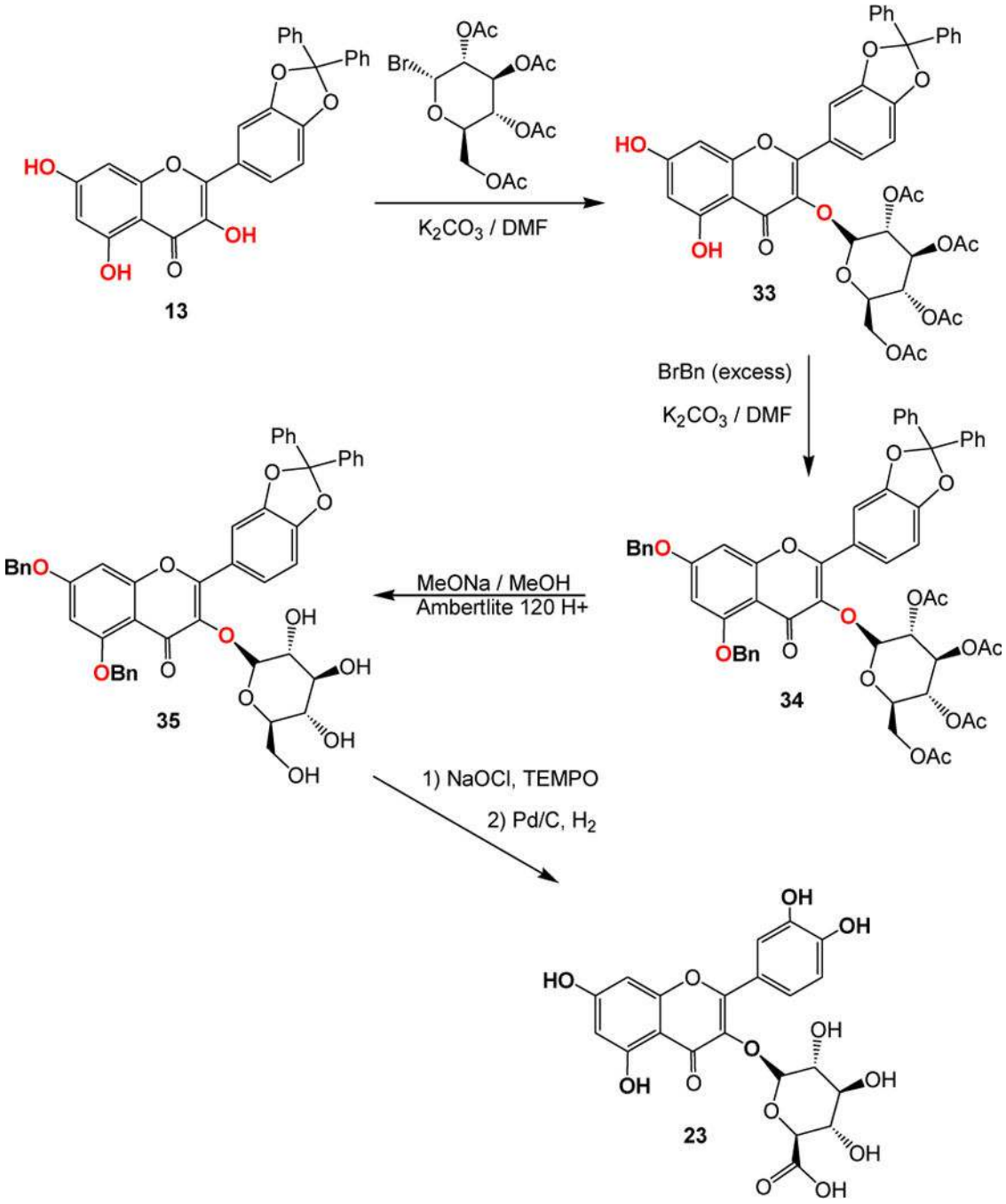

Figure 14.
Synthesis of quercetin-3-*O*-β-D-glucuronide **23**.

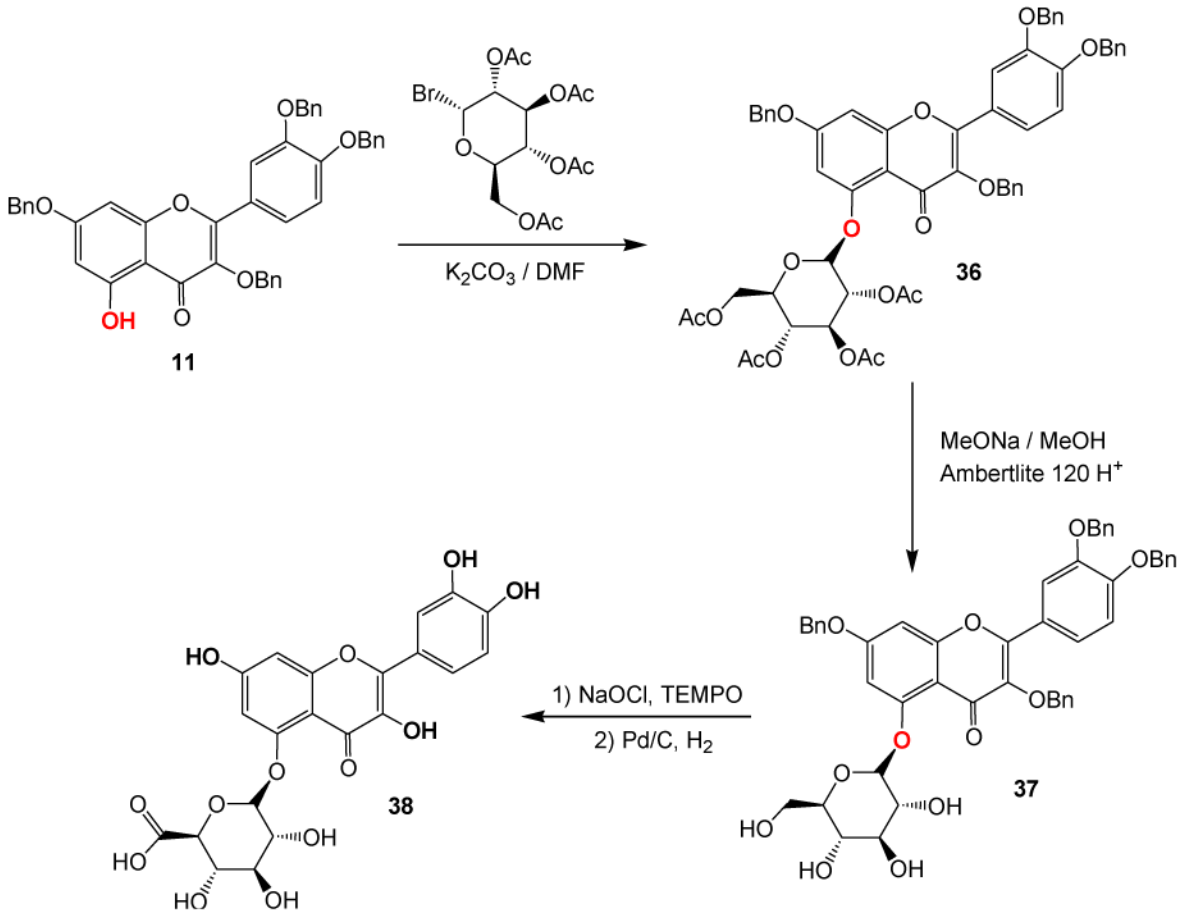

Figure 15.
Synthesis of quercetin-5-*O*-β-D-glucuronide **38**.

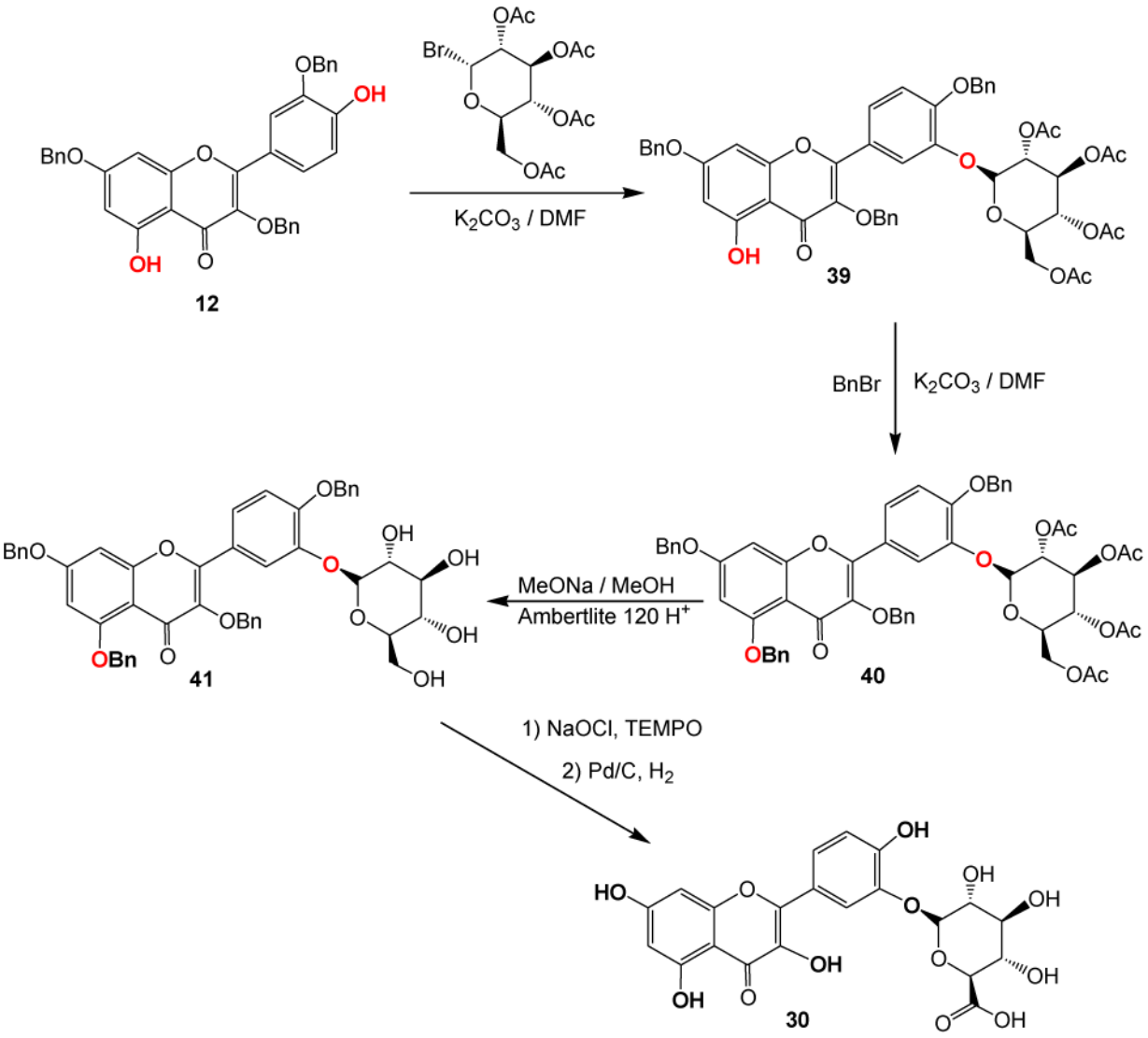

Figure 16.
Synthesis of quercetin-3′-*O*-β-D-glucuronide **30**.

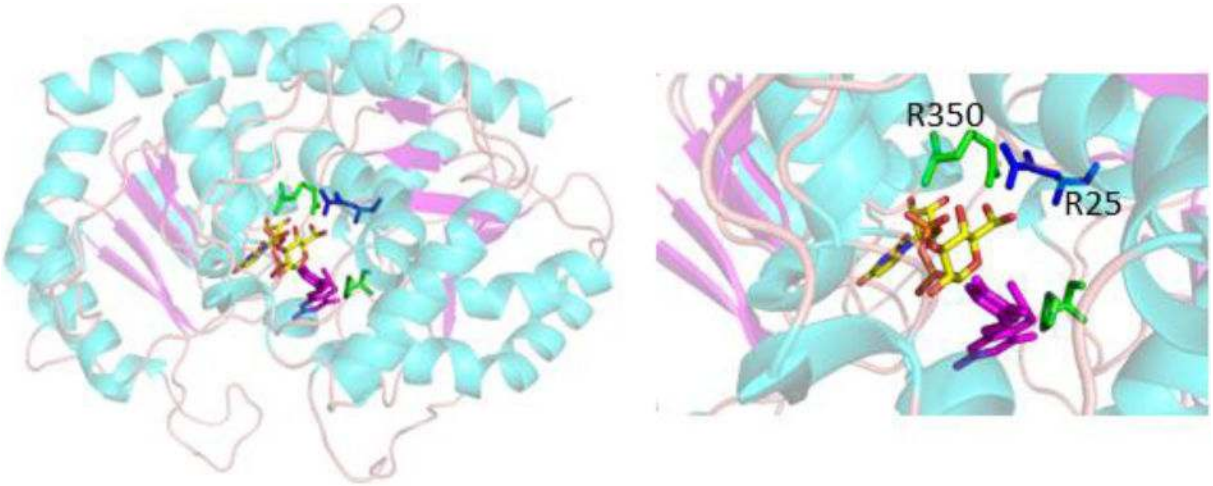

Figure 17.

A modeled structure of the flavonoid 7-*O*-glucuronosyltransferase UGT88D7. The docked UDP-glucuronic acid (in yellow) and epicatechin (magenta) are shown as bond models. The key amino acids Arg350 in UGT88D7 and Arg25 in UGT94B1 are also shown as bond models in green and blue, respectively.

Table 1

Properties of Human Glucuronosyltransferases. Regioselectivity shows products formed from luteolin (L), quercetin (Q), or epicatechin (E).

Isoenzyme	Tissue localization	Preferred substrates	Regioselectivity	Reference
UGT1A1	Liver, intestine	Bilrubin; anthraquinones; oripavin opiods (e.g.buprenorphine); estrogens; phenols and flavonoids (e.g chrysin, apigenin, baicelin, luteolin, quercetin, fisetin, genistein, narigenin).	L-4′-O-GlcA > L-3′-O-GlcA>L-7-O-GlcA Q-3′-O-GlcA > Q-4′-O-GlcA> Q-7-O-GlcA	16, 126, 157, 158
UGT1A3	Liver	Certain estrogens; flavonoids; coumarin; amines;anthraquinones.	L-7-O-GlcA > L-4′-O-GlcA > L-3′-O-GlcA Q-3′-O-GlcA > Q-7-O-GlcA>Q-3-O-GlcA > Q-4′-O-GlcA	16, 159
UGT1A4	Liver	Primary, secondary, and tertiary amines; monoterpenoid alcohols; sapogenins;androstanediol; progestins; certain flavonoids.	L-7-O-GlcA>L-4′-O-GlcA>L-3′-O-GlcA Q-4′-O-GlcA>Q-3′-O-GlcA	16, 160
UGT1A6	Liver, intestine, kidney	Few planar phenolic compounds and some flavonoids.	L-7-O-GlcA Q-4′-O-GlcA > Q-7-O-GlcA>Q-3′-O-GlcA > Q-3-O-GlcA	16, 161
UGT1A8	Kidney, colon, intestine, liver	Flavonoids including apigenin, luteolin, narigenin, daizdein.	L-7-O-GlcA>L-3′-O-GlcA>L-4′-O-GlcA Q-3′-O-GlcA> Q-7-O-GlcA>Q-4′-O-GlcA > Q-3-O-GlcA	16, 126
UGT1A9	Liver, kidney	Flavonoids; anthraquinones; bulky phenols; certain aliphatic alcohols; nonsteroidal anti-inflammatory drugs.	L-4′-O-GlcA > L-3′-O-GlcA>L-7-O-GlcA Q-3′-O-GlcA>Q-4′-O-GlcA > Q-7-O-GlcA EC-3′-O-GlcA > EC-5-O-GlcA 3′-O-Me-EC-5-O-GlcA	16, 161 131, 132
UGT1A10	Intestine, liver	mycophenolic acid; some flavonoids; antineoplastic and immunosuppressive agents.	L-7-O-GlcA > L-4′-O-GlcA> L-3′-O-GlcA Q-7-O-GlcA> Q-3-O-GlcA > Q-4′-O-GlcA	16, 162
UGT2B7	Kidney, liver	Some flavonoids	L-3′-O-GlcA Q-7-O-GlcA > Q-3′-O-GlcA > Q-3-O-GlcA	16
UGT2B15	Intestine, bone marrow and immune system, liver	Some flavonoids	L-7-O-GlcA > L-4′-O-GlcA > L-3′-O-GlcA Q-7-O-GlcA > Q-4′-O-GlcA > Q-3′-O-GlcA	16