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Biochemical synthesis of coumarin glycosides: A review†

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Coumarin glycosides have shown immense potential for diverse biological activities and have been explored extensively as highly prospective biomolecules. In recent years, coumarin glycosides have been used as enzyme inhibitor molecules, environmentally-sensitive fluorescent sensors, the building material of single-excitation and dual emission graphene composite, fluorophore-tagged glycosides, molecule to assay enzyme activity on model bacterial strains, the nucleoside monomer unit for the photoswitchable formation of a DNA interstrand cross-link along with many other applications. In this review, we have compiled biochemically afforded coumarin glycosides obtained *via* mutagenic synthesis, chemo-enzymatic synthesis and hairy root culture system synthesis. This review will play the role of a reservoir of biochemically synthesized glycosylated coumarins and encourage medicinal chemists to explore the potential of these molecules as drug candidates and further biological applications.

Keywords: Coumarins, coumarin glycosides, mutagenic synthesis, chemoenzymatic synthesis.

1. Introduction

Coumarins are heterocycles consisting of fused benzene and α-pyrone rings, which form a very significant class of natural products. After its first isolation from tonka beans, more than thirteen hundred types of coumarins have been isolated and identified as secondary metabolites from bacteria, fungi and green plants[1,2]. These naturally occurring compounds had displayed a wide array of biological and pharmacological activities, which encouraged scientists to synthesize their analogues. With an aim to produce analogue series, divergence was introduced in the basic coumarin moiety by various substitutions in the aromatic ring and 3- and 4-positions of benzopyran ring of the coumarin. Nevertheless, structural biodiversity in coumarin glycosides arises from the attachment of the sugar moieties at specific positions of the aglycon core. When attached to the drug molecules, they manoeuvre their solubility, membrane transport, pharmacokinetics and pharmacodynamics[3]. When used as a drug molecule, these sugar modules contribute towards the molecular recognition of their cellular target and help the aglycon part to affix with DNA strand by anchoring with its major or minor groove[4]. Biological relevances of the coumarin glycosides have inspired biochemists and microbiologists to develop various chemical, chemoenzymatic, enzymatic and engineered biosynthesis methodologies to fabricate coumarin glycosides. However, exploration of literature conceded that review articles on naturally occurring or synthesized coumarins[5–9] are not so plentiful in literature and moreover, coumarin glycosides were recorded as an insignificant part therein. On the other hand, the use of whole-cell fermentation (wild type/mutant) or application of isolated and purified natural-product enzymes have been found to be advantageous in comparison to classical methods of total synthesis of natural products which are often lengthy and afford very poor overall yield. In the modern manufacturing methodologies of natural products, fermentation processes are used widely for the manufacturing of mass production of natural products, such as antibiotics, vitamins and other

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biomolecules. Through improved strains, metabolic regulation and optimisation of fermentation processes, these natural products are afforded with low cost and high product quality. This is a primary review, where we have emphasized exclusively on the biochemical synthesis of coumarin glycosides so that the researchers working in this field could engross their attention towards these biochemical methodologies and further explore and flourish this research area.

1.1. Glycosylating sugars

The coumarin glycosides obtained from biosynthesis, chemoenzymatic procedure and microbial source contain a variety of sugar moieties attached to coumarins. In this part of the review, we included all the sugar structures affixed in different coumarin glycosides found in the literature. General convention was followed for the numbering of the sugar rings (**1**-**7**, Fig. 1).

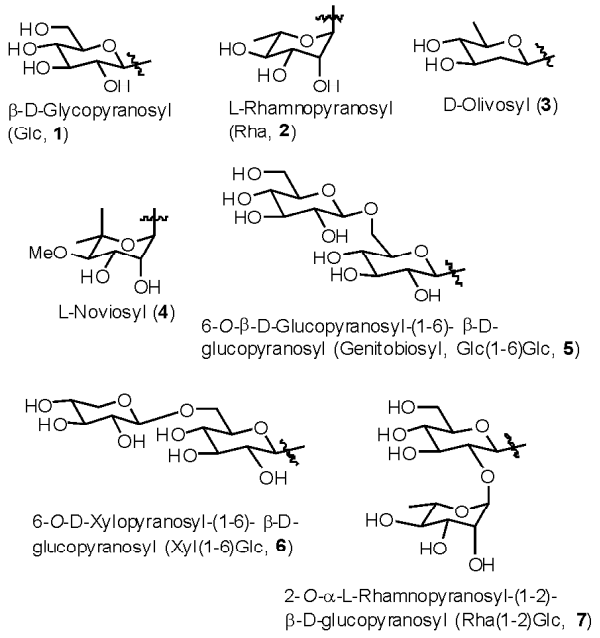


Fig. 1. Sugar moieties associated with coumarin glycosides described in this article.

1.2. Glycosylating enzymes

Glycosidic bond formation reaction between the sugar moiety and aglycone is catalyzed by the glycosyltransferases (GTs, EC 2.4.x.y), which is a diverse group of enzymes present in nature. Glycosidation reaction provides an infinite variety to the structural diversity of the plant secondary metabolites and it is also involved in the biosynthesis of glycolipids, glycoproteins, polysaccharides and secondary metabolites. These enzymes are able to alter the hydrophobicity, stability, chemical properties, subcellular localization and bioactivity of the acceptors, which includes plant metabolites, phytotoxins and xenobiotics[10]. The GTs are classified into GT-A and GT-B depending on the Rossmann fold which is based on the classic structural motif of the nucleotide binding domain as a basic unit to categorize the structures[11,12]. The GT-A contains a central β-sheet surrounded by α-helices and make β/α/β combination type to form Rossmann fold like structure. The general characteristic of the enzymes in this family is the presence of common Asp-X-Asp (DXD) motif which interacts with phosphate groups of nucleotide donor by coordinating with a divalent cation Mn^{2+}, which is a prerequisite for the enzyme activity[13].

The enzymes of the GT-A family have two well conserved regions, one of which is Rossmann-type nucleotide binding domain consisting of 100–120 amino acid residues and terminated by the DXD motif. The second one is the part of the active site corresponding to the region β6-α4-α5, which interact with both the donor sugar and acceptor sugar. The GT-B includes two separate Rossmann like fold domains, which are connected by a linker. The major difference between these two categories is that the Rossmann domains are adjoining in case of GT-A and they are linked by a flexible linker in GT-B[13].

Along with these two families of glycosyltransferases, two additional superfamilies were also identified. The third type of glycosyltransferases was predicted on the basis of iterative sequence search by using EC-BLAST (Enzyme Commission-Basic Local Alignment Search Tool) programme and was coined as GT-C[14]. The final superfamily GT-D includes all miscellaneous type of glycosyltransferases which do not fit in any other category[15–17].

Freemont and co-workers[18] reported the crystal structure of DNA modifying bacteriophage T4-glucosyltransferase enzyme in the presence and absence of its substrate uridine diphosphoglucose. It was the first report of the crystal structure of any glycosyltransferase enzyme and was assigned as the structure of GT-B. Since then, numerous crystal structures corresponding to different glycosyltransferases from prokaryotes and eukaryotes have been depicted. The structure of nucleotide-diphospho-sugar transferase (SpsA) in

complex with both Mg-UDP and Mn-UDP were obtained from *Bacillus subtilis* and helped to illustrate the reaction mechanism of glycosyltransferases[19]. Relative analysis of these crystal structures divulged that adaptive binding of the disaccharide unit of the acceptor sugar assist the enzyme to undergo an inverted S_{N2} type displacement or a retaining S_{Ni} like reaction[20].

Glycosyltransferases have an enormous potential to be applied in the pharmaceutical biotechnology due to their relative stability, susceptibility to genetic engineering and broad substrate specificity[21]. Numerous GTs have been found to be suitable enough to alter the glycosylating pattern. However, the restriction of the substrate specificity of the enzymes has become a limiting factor for the diversification of natural products. Biogenetic engineering is fulfilling this gap by developing GTs with defined specificities which will enable us to diversify the library of novel glycosylated compounds. The viability of engineering strategy (*in vivo*) and chemoenzymatic synthetic approach (*in vitro*) has inculcated new efforts in the field of glycodiversification and will enable researchers to cultivate alternative sugar modified structures of the natural products and potent drug molecules[22].

2. Mutagenic synthesis

Mutagenesis is a sophisticated technique of molecular biology, where specific and intentional alteration to the DNA sequence of a gene is carried out to investigate structural and functional activity of DNA, RNA and protein molecules. Site-directed mutagenesis has emerged as an important tool for the introduction of mutation in DNA sequences.

Among various classes of coumarin glycosides, one of the major classes is constituted by aminocoumarins, as they have been found to be the source of a large number of natural product antibiotics[23]. With an aim to screen a maximum number of analogues of their derivatives, these compounds have been explored extensively through mutagenic synthesis processes. Three most important and classical aminocoumarins, such as novobiocin (**8**), clorobiocin (**9**) and coumermycin A1 (**10**) has a common structural feature which contains an aromatic acyl component, a 3-amino-4,7-dihydroxycoumarin moiety and a deoxysugar L-noviosyl (4-*O*-methyl-5-*C*-methyl-L-rhamnose) acylated at C-3 hydroxyl with either a carbamoyl or a 5-methyl-pyrrole-2-carboxyl moiety (Fig. 2). These compounds are natural products of ubiquitous soil-dwelling bacteria, *Streptomycetes*. The complete genome sequence of several *Streptomyces* are known in literature[24,25] and the wild type producer strains for these antibiotic compounds were found to be *Streptomyces caeruleus* (Syn. *Streptomyces spheroids*), *Streptomyces roseochromogenes* and *Streptomyces rishiriensis*. These antibiotics are capable of interacting with the B subunit of bacterial DNA gyrase to inhibit ATPase related activity of the enzyme[26]. DNA gyrase has become a conventional and ideal target for drugs due to its availability in all bacteria and non-existence in humans[27,28].

Following the development in the field of molecular cloning systems in *Streptomyces*, the isolation of biosynthetic genes for antibiotic producing members of this genus became achievable. These clones were used to fabricate novel antibiotics through the transfer of biosynthetic genetic material between different antibiotic producing *Streptomyces*. Hopwood and Malpartida[29] carried out molecular cloning of the entire biosynthetic pathway of *Streptomyces* antibiotic and the way it is expressed in a heterologous host. Later the same research group reported production of antibiotics like actinorhodin (**11**), granaticin (**12**) and medermycin (**13**) by genetic engineering, which is considered as the first account for the production of hybrid antibiotics[30]. Simocyclinones (**14**), the potential inhibitors of bacterial gyrase were also synthesized by mutant genes expressed in *Streptomyces lividans* T7[31].

In the recent past, significant advancement has been achieved in the generation of new aminocoumarin antibiotic by biosynthetic pathways. The biosynthetic gene clusters of these compounds have been identified and the effectiveness of these genes have been studied. With the extensive understanding of these pathways and adequate knowledge to plan out the rational design of products through cloning of the biosynthetic gene clusters of the corresponding aminocoumarins, researchers are competent to produce new antibiotics of this class with retained DNA gyrase inhibitor activity and improved pharmacokinetics. In this approach, genetic combinatorial experiments combined with the extent of organic synthesis afforded a wide range of aminocoumarin antibiotics.

The entire DNA sequence of the biosynthetic gene clusters of three major aminocoumarins, *i.e.* novobiocin (**8**)[32],

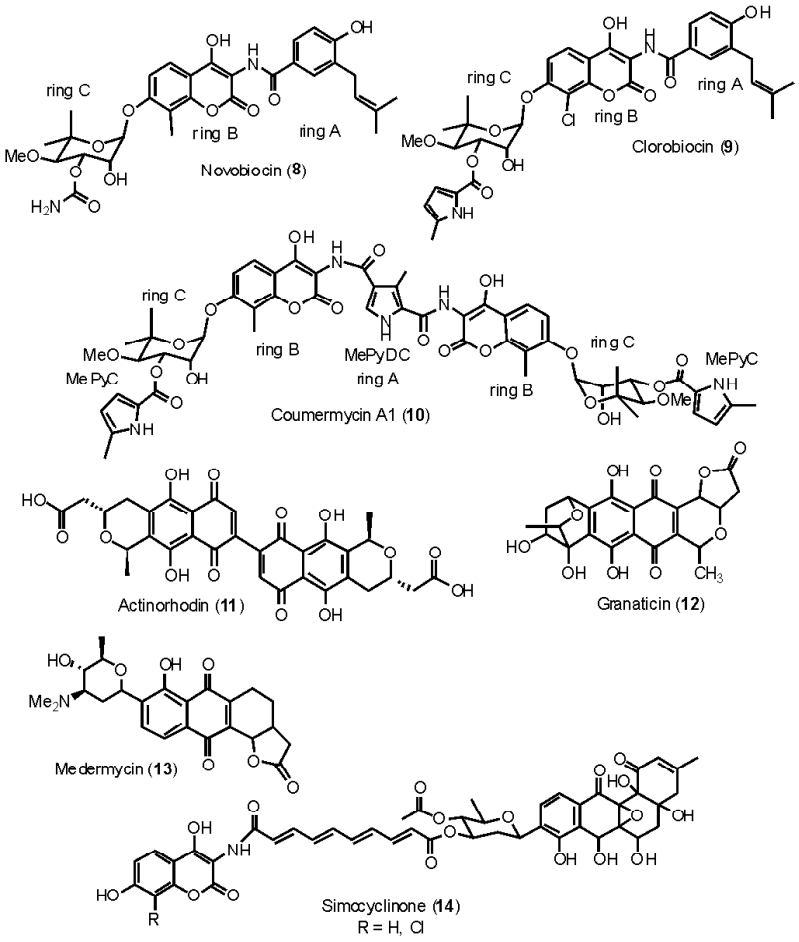


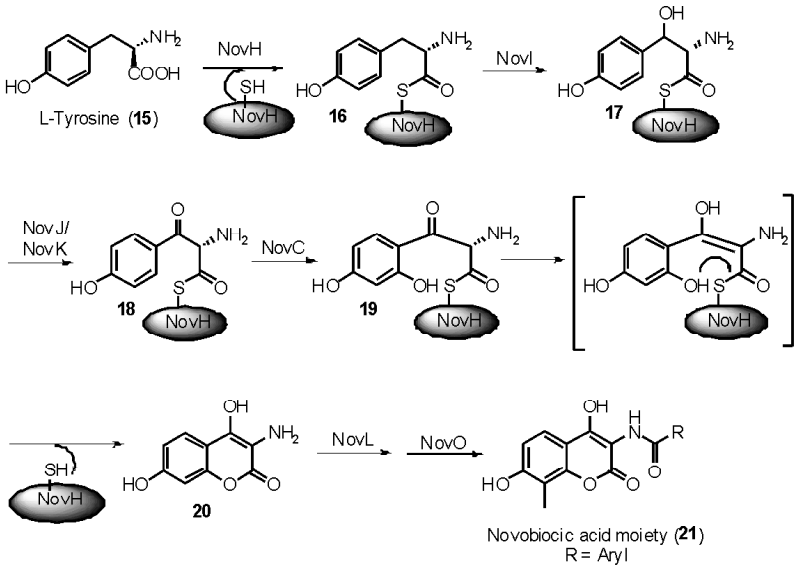
Fig. 2. Important antibiotics synthesized by genetically engineered microbes.

clorobiocin (**9**)[33] and coumermycin A1 (**10**)[34] are already known in literature. The functional analysis of most of these genes contained in these clusters were elucidated by biochemical experiments and gene inactivation[35].

2.1. Synthesis of aminocoumarin moiety

Biochemical experiments revealed that the gene products of *novHIJK* were responsible for the biosynthesis of aminocoumarin moiety, where L-tyrosine (**15**) was activated by the adenylation domain of the 65 kDa protein NovH (Scheme 1)[36,37]. This entity was affixed with the phosphopantetheinyl cofactor of a peptidyl carrier protein (PCP) domain of NovH through a thioester linkage. Next, enzyme bound tyrosine was found to undergo a monooxygenase reaction which is hydroxylated by a cytochrome P450 monooxygenase NovI (45 kDa heme protein) to produce (2*S*,3*R*)-β-hydroxy-tyrosyl-S-NovH (**17**). The oxidoreductase enzyme NovJ/NovK oxidizes this intermediate into β-keto-tyrosyl-S-NovH (**18**), where NADP played the role of an electron acceptor[37]. The final steps of this biosynthetic procedure, *i.e.* the cyclization reaction in aminocoumarin are not elucidated yet, but it was speculated as the hydroxylation at *ortho*-position of the phenyl ring by NovC and methylation at the *meta*-position of the phenyl ring by NovO (comparable to some quinine *C*-methyltransferases)[37,38]. It is considered that the intramolecular nucleophilic attack of the *ortho*-hydroxyl group (of compound **19**) on the carbonyl centre of the thioester cyclise the moiety to afford the coumarin ring (**20**) and to regenerate NovH. However, later studies provided evidence that NovC is not required for the biosynthesis of the aminocoumarin moiety of novobiocin[39].

Analogous to the part of a single operon structure *novHIJK* in novobiocin, the clusters of clorobiocin and coumermycin



Scheme 1. Biosynthesis of aminocoumarin moiety.

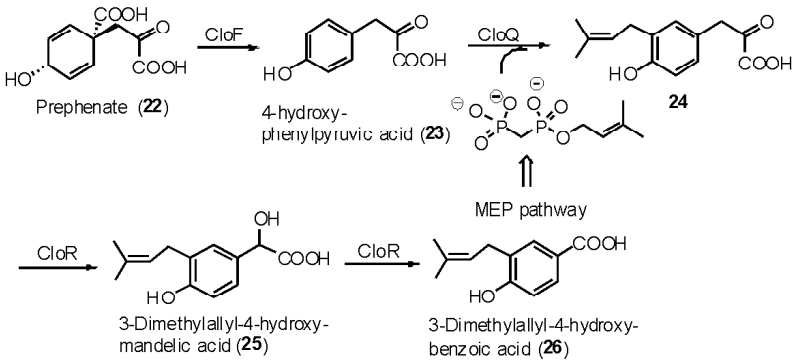
A1 contained *cloHIJK* and *couHIJK* respectively, which were found to carry out the same reaction sequence in the biosynthetic pathway of their corresponding aminocoumarin moieties. The simocyclinone cluster had orthologs of these genes, which were found to be *simJ* and *simI* (adjacent to each other), and genes *simK* and *simJ1* (situated in different positions of *simHI*)[40].

The biosynthetic gene cluster of clorobiocin (**9**), coumermycin A1 (**10**) and simocyclinone (**14**) contained small open reading frames known as *cloY*, *couY* and *simY*, respectively. Feeding experiment of the inactivated *cloY* mutants with 3-amino-4,7-dihydroxycoumarin showed that *cloY* is necessary for the formation of the aminocoumarin moiety in the clorobiocin molecule[41]. Methylation at C-8 position of novobiocin and coumermycin, was catalyzed by NovO and CouO[42,43] and the reaction occurred after the amidation reaction between C-3 amine and carboxylic moiety. Clorobiocin (**9**) and simocyclinone D8 (**14**, R = Cl) molecules contain chlorine atoms at the C-8 position. Genetic studies showed that the FAD-dependent halogenase Clo-hal is responsible for the introduction of this halogen[44]. Feeding studies *in vivo* of intermediates to mutant stains indirectly demonstrated that amide formation precedes halogenations[45].

2.2. Synthesis of prenylated 4-hydroxybenzoylated moieties

Novobiocin (**8**) and clorobiocin (**9**) molecules contain a 3-prenylated 4-hydroxy-benzoyl moiety attached with C-3 amine via an amide bond. This acyl moiety is prenylated by an unusual prenyltransferase, CloQ in the *meta*-position of the aromatic ring, using dimethylallyl diphosphate as a prenyl donor[46]. Regular prenyltransferases were found to be absent in the biosynthetic gene cluster of these two antibiotics and monomeric protein CloQ was found to catalyze the *C*-prenylation of different aromatic substrates (Scheme 2)[47,48]. The gene cluster of clorobiocin restrained a prephenate dehydrogenases CloF, which converted prephenate (**22**) into 4-hydroxy phenylpyruvate (**23**) and delivered as the aromatic substrate for the succeeding reaction with prenyltransferase CloQ. The isoprenoid substrate for the *meta*-substitution on 4-hydroxy phenylpyruvate (**24**) is dimethylallyl diphosphate (DMAPP), which came from the methylerythritol phosphate (MEP) pathway[49,50]. Next, the prenylated product (3-dimethylallyl-4-hydroxymandelic acid, **25**) was converted into 3-dimethylallyl-4-hydroxybenzoic acid (**26**) by the bifunctional non-heme Fe(II)-dependent dioxygenase CloR in two consecutive decarboxylation reactions[51]. Orthologs of these genes were also found in the novobiocin gene cluster as *novF*, *novQ* and *novR*, which carried out the corresponding reactions[32].

Coumermycin A1 (**10**) contained a 3-methyl-pyrrole-2,4-dicarboxylic acid unit at the C-3 amine position and accord-

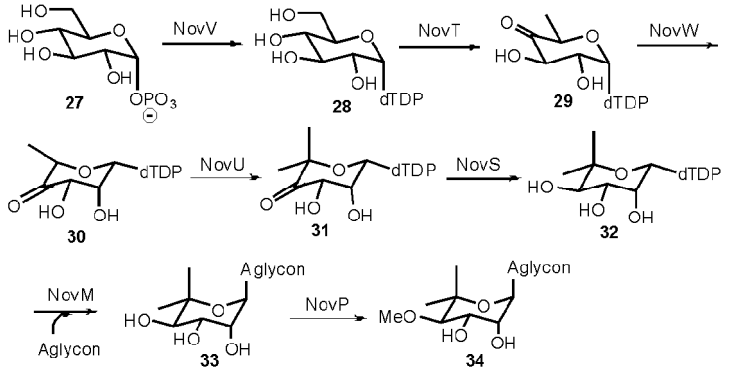


Scheme 2. Biosynthesis of prenyl moiety.

ingly, the biosynthetic gene cluster of this antibiotic showed the lack of orthologs of *cloF*, *cloQ* and *cloR*. This gene cluster showed a group of genes named as *couR1* to *couR4*, which were not orthologs in novobiocin and clorobiocin and supposed to direct the biosynthesis of central pyrrole moiety of coumermycin A1[52].

2.3. Synthesis of deoxy sugar moieties

Novobiocin (**8**), clorobiocin (**9**) and coumermycin A1 (**10**) contain the common deoxysugar noviose (5-methyl-L-rhamnose/4-*O*-methyl-5-*C*-methyl-L-rhamnose). This sugar unit is linked to C-7 hydroxyl of the aminocoumarin moiety and is acylated at its C-3 hydroxyl either with a carbamoyl or with a 5-methyl-pyrrole-2-carboxyl moiety. Biosynthetic pathway for L-rhamnose is well known from various organisms[53] but the presence of the methyl group at C-5 produces a unique 5,5-*gem*-dimethyl structural moiety. Biosynthetic pathway for dTDP-5-*C*-methyl-L-rhamnose moiety starting from glucose-1-phosphate (**27**) was proposed based on the feeding experiments, which indicated that one of these two methyl groups at C-5 is derived from *S*-adenosylmethionine[54]. A group of five genes *novSTUVW* in novobiocin cluster directed the first five steps of biosynthesis of this sugar moiety converting it into dTDP-5-methyl-L-rhamnose (**32**) starting from glucose-1-phosphate (**27**, Scheme 3)[55,56]. Orthologs of these genes in clorobiocin and coumermycin cluster carry out the same reaction sequences to produce antibiotics clorobiocin (**9**) and coumermycin A1 (**10**), respectively[57,58]. The dTDP activated deoxysugar **32** is added to the aglycon moiety by glycosyltransferase NovM[59,60] followed by methylation at the C-4 hydroxyl of the sugar unit by NovP to afford structural moiety **34**. In clorobiocin (**9**) and coumermycin (**10**) gene clusters, these reactions have been carried out by CloM and CloP, and CouM and CouP, respectively[61,62]. In novobiocin (**8**), the concluding reaction of this pathway is the attachment of the carbamoyl group to the C-3 hydroxyl of the deoxysugar moiety by a reaction catalyzed by NovN[63,64].



Scheme 3. Biosyntheis of deoxysugar moiety.

2.4. Synthesis of pyrrole moiety

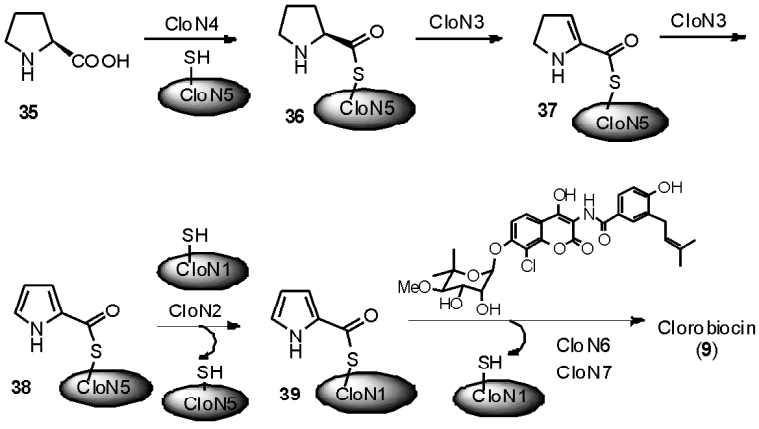
In the deoxy sugar unit (ring C) of clorobiocin and coumermycin A1, the acyl group attached at the C-3 hydroxyl is 5-methyl-pyrrole-2-carboxyl. In clorobiocin, this pyrrole moiety is formed from proline moiety by the catalysis of CloN3, CloN4 and CloN5. Initially, the L-proline (**35**) is activated by acyl adenylation domain enzyme CloN4 and mounted as thioester on the phosphopantetheinyl cofactor of the peptidyl carrier protein (PCP) subunit CloN5 (**36**, Scheme 4)[65,66]. Orthogonal genes have been identified in the gene cluster of coumermycin A1 moiety as *couN3*, *couN4* and *couN5*. Next, the prolyl-S-PCP unit is oxidized by the flavoprotein dehydrogenase CloN3 into a pyrrole derivative. In this biosynthetic pathway, CloN1 has been identified as a second acyl carrier protein. The pyrrole moiety on CloN5 gets transferred to the CloN1 by the catalytic activity of acyltransferase CloN2 prior to its attachment with C-3 hydroxyl of L-noviose moiety by catalytic action of acyltransferase CloN7[67,68]. Gene cluster of coumermycin A1 also contain orthogonal genes for the consecutive steps, which were recognized as *couN1*, *couN2* and *couN7*[69,70]. At the final stage of the synthesis, 5-methyl group get attached to pyrrole moiety by the catalytic activity of methyl transferase CloN6[71,72]. Nevertheless, methylation reaction has also been hypothesized to take place in a concerted way in the presence of both CloN6 and CloN7.

Earlier research work suggested that the central 3-methylpyrrole-2,4-dicarboxylic acid moiety (CPM) of coumermycin A1 is formed through a biosynthetic pathway unlike the pathway for the synthesis of terminal 5-methyl-pyrrole-2-carboxylic moieties[67]. The minimal set of genes required for the generation of CPM scaffold has been identified as a group of five genes *couR1*, *couR2a*, *couR2b*, *couR3* and *couR4* which are assembled together in a contiguous 4.7 kb region in the biosynthetic gene cluster of coumermycin A1[52]. It was also established that the genes *couR1-couR4* are sufficient to obtain the CPM moiety and adjoining genes *couR5* and *couR6* are not essential for the biosynthetic pathway.

2.3. Enzymes involved in linking aminocoumarin constituents

The amide group at the C-3 position of the 3-amino-4,7-dihydroxyaminocoumarin moiety is a link between the aminocoumarin moiety and the acyl moiety. The amide synthetase enzymes accountable for the formation of this amide bonds in novobiocin (**8**), clorobiocin (**9**), coumermycin (**10**) and simocyclinone (**14**) are identified as NovL[73], CloL[74], CouL[61,75] and SimL[31,76], respectively. These monomeric proteins catalyze the activation of the acyl substrate by forming an acyl adenylate and transfer the activated acyl substrate to the amine group. The substrate specificity of these amide synthetases has been found to be different and this feature has been exploited for the mutagenetic generation of new derivatives of aminocoumarin antibiotics.

After the amide bond formation between 3-amine aglycon and acyl moieties, the aglycon moieties of novobiocin, clorobiocin and coumermycin gets methylated or chlorinated at the C-8 position under catalytic activity of NovO, CouO and Clo-hal, respectively. Next, the glycosyltransferases NovM[77] and CouM[62] transfer the 5-*C*-methyl-L-rhamnosyl



Scheme 4. Biosynthesis of pyrrole moiety.

moieties onto the C-7 hydroxyl group of these aglycons in novobiocin and coumermycin, respectively. After the attachment of this carbohydrate moiety, its 4-hydroxyl group gets methylated by S-adenosyl-methionine (SAM)-dependent methyltransferases NovP[78], CouP[62] and CloP[61], respectively.

The carbamoylation reaction of C-3 hydroxyl group in deoxysugar moiety is catalyzed by NovN in novobiocin. In case of coumermycin and clorobiocin, the C-3 hydroxyl of deoxysugar has a 5-methyl-pyrrole-carboxyl moiety. Biochemical investigation proposed that CloN2 could be responsible for transferring the pyrrole-2-carboxyl moiety from the acyl carrier protein CloN5 to the hydroxyl group of the deoxysugar, followed by methylation of C-5 of the pyrrole moiety. However, some literature suggested that the C-5 methylation and transfer of the pyrrole-2-carboxyl moiety could happen simultaneously[71].

3. Chemoenzymatic synthesis

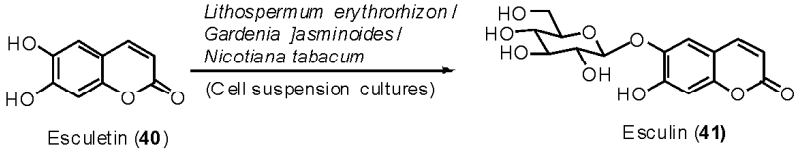
Chemoenzymatic synthesis strategy has been defined as a process where either the glycosyl moiety has been attached with the basic coumarin moiety by using enzymes (Schemes 5, 8, 9, 10, 11 and 13) or the glycosyl moiety of the coumarin glycosides have been diversified by enzymatic reactions (Schemes 6, 7 and 12). This process of synthesizing coumarin glycosides or aminocoumarin antibiotics has the potential to generate a very large range of structural diversity in the molecules. A foremost drawback in the mutagenic synthesis of these antibiotics is the suitability of the synthetic substrate, where the substrate molecules could be rejected by the bacteria across its cell wall. On the other hand, some of these substrates may not be stable enough in the cell culture conditions. In these conditions, chemoenzymatic synthesis offers an alternative option.

Cell suspension cultures of *Lithospermum erythrorhizon*, *Gardenia jasminoides* and *Nicotiana tabacum* were screened for the glycosylation reaction of esculetin (**40**) to convert it into esculin (**41**) (Scheme 5)[79]. Five different culture strains of *L. erythrorhizon* were tested and one of the culture strains (C-144-7-6) was found to be superior for glycosylation which converted 40 to 50% of esculetin into its glycosylated analogue within 24 h. It was found that the rate of glycosylation depends on the stage of cell growth, medium composition such as growth hormones and sugar.

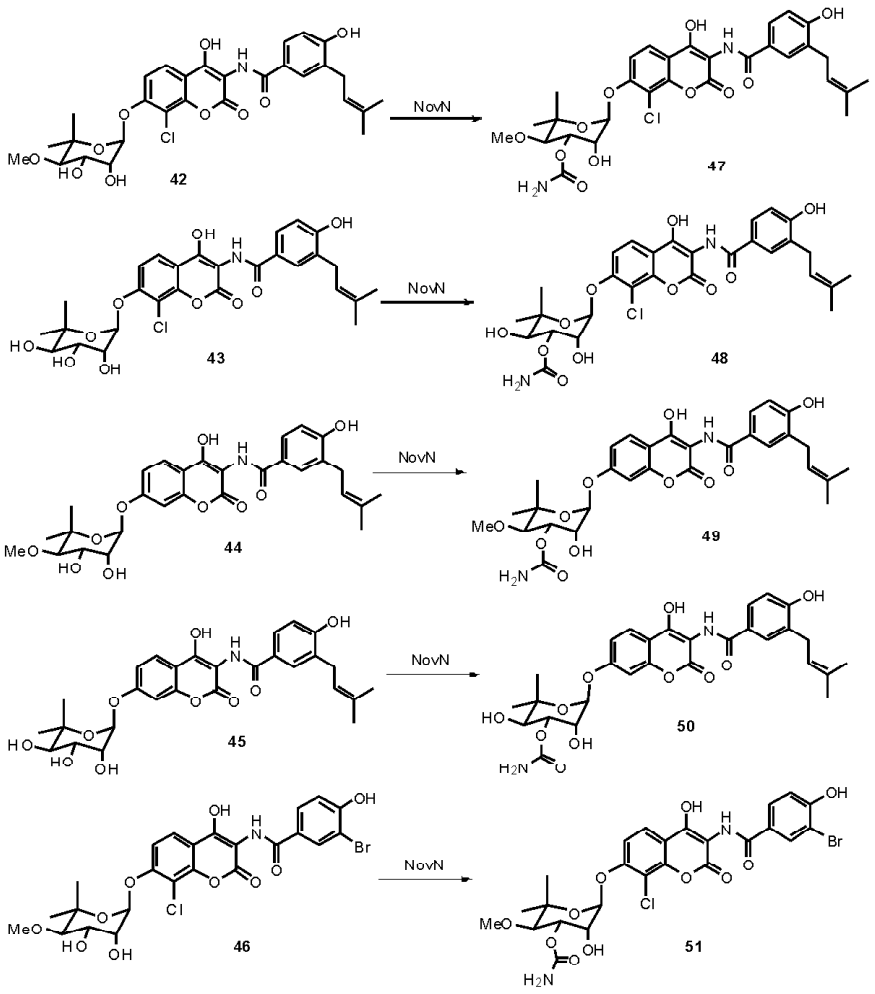
The carbamoylation of five aminocoumarin antibiotics novclobiocin 104 (**42**), novclobiocin 105 (**43**), novclobiocin 107 (**44**), novclobiocin 108 (**45**) and novclobiocin 283 (**46**) was carried out following enzymatic synthetic procedure[63]. The carbamoyltransferase NovN was used to produce new aminocoumarin antibiotics novclobiocin 114 (**47**), novclobiocin 115 (**48**), novclobiocin 117 (**49**), novclobiocin 118 (**50**) and novclobiocin 284 (**51**), respectively having carbamoyl moiety at the C-3 position of the sugar ring of the corresponding antibiotics (Scheme 6).

In clorobiocin (**9**) and coumermycin A1 (**10**) antibiotics, the key pharmacophore has been identified as 5-methyl-2-pyrrolylcarbonyl moiety, which targets the ATP-binding site of GyrB. Garneau-Tsodikova and co-workers[80] introduced structural diversity at the C-3 position of the noviosyl ring with heterocyclic acyl groups to generate a series of novel and more potent coumarin antibiotics (**53**-**76**). These heterocyclic acyl groups were transferred to the C-3 hydroxyl of the noviosyl moiety by acyltransferase CloN7 or CouN7 from the carrier protein CloN1 or CouN1 (Scheme 7). 5-Methylthiophene novobiocin (**59**) was scaled up to milligram quantity for screening its activity against Gram-negative bacteria *Bacillus subtilis* PY79 and *Bacillus cereus* ATCC 14893 where it showed activity of the same magnitude as that of novobiocin (**8**). However, the novobiocin analogue **59** was found to be less effective against Gram-positive bacterial strains wild-type *E. coli* MC4100 and the mutated *E. coli* NR698 imp4213.

Due to the stringent specificity of glycosyltransferases, Williams *et al.*[81] reported the use of a simple high-throughput screen based on a fluorescence surrogate acceptor substrate and expanded the promiscuity of a natural product GT



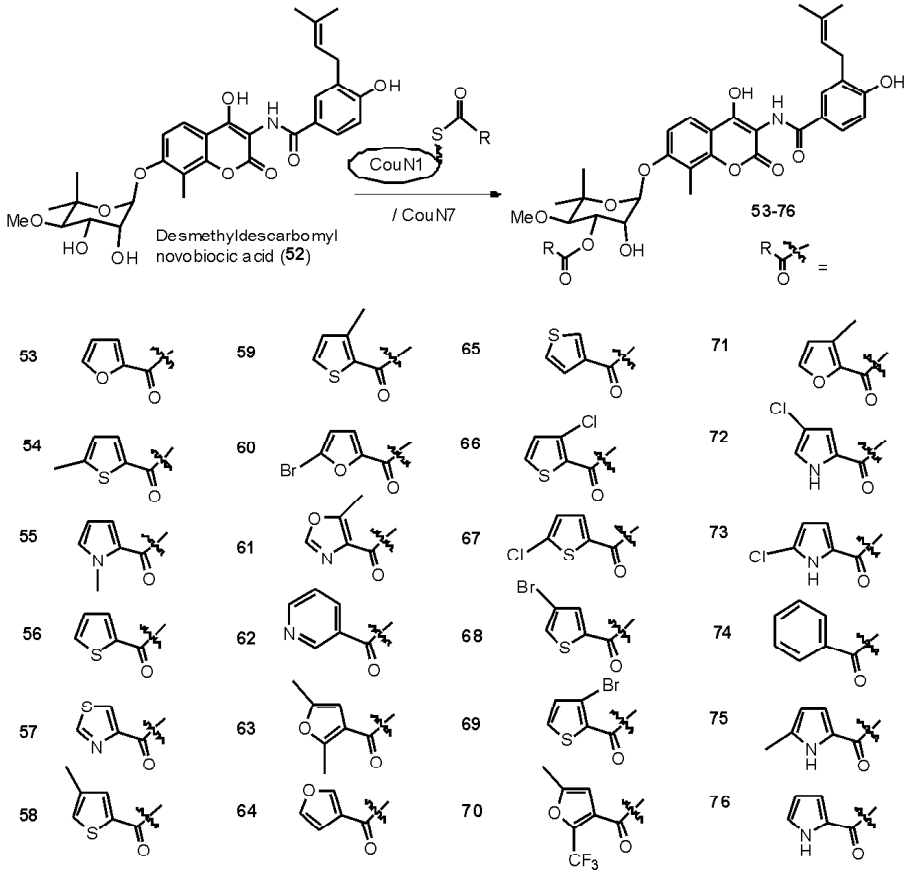
Scheme 5. Glycolysation of esculetin (**40**) by cell suspension cultures.



Scheme 6. Carbamoylation of aminocoumarin antibiotics.

(oleandomycin GT) via directed evolution to glycolrandomize a vast range of therapeutically important acceptor molecules (**81**-**84**). Triple mutant P67T/S132F/A242V showed enhanced glycosylation activity, which was around thirty fold higher reactivity than that of the reaction between 4-methylumbelliferone (**77**) and uridine diphosphate glucose (UDP-Glc) catalyzed by wild type OleD (WT OleD). This triple mutant was constructed after identification of three clones 2C3 (single amino acid mutation A242V), 8B3 (double amino acid mutation P67T/I112T) and 7B9 (double amino acid mutation S132F/G340W) from the library of OleD mutants to be more active than wild-type OleD (WT OleD). However, G340W and I112T mutations from 7B9 and 8B3, respectively were found to be non-functional. The wild-type OleD accepted aminocoumarin (**80**) as a substrate at a very slow rate compared to its natural substrate oleandomycin, whereas the activity of the triple mutant P67T/S132F/A242V towards small phenolics was high compared to the wild-type OleD (Scheme 8).

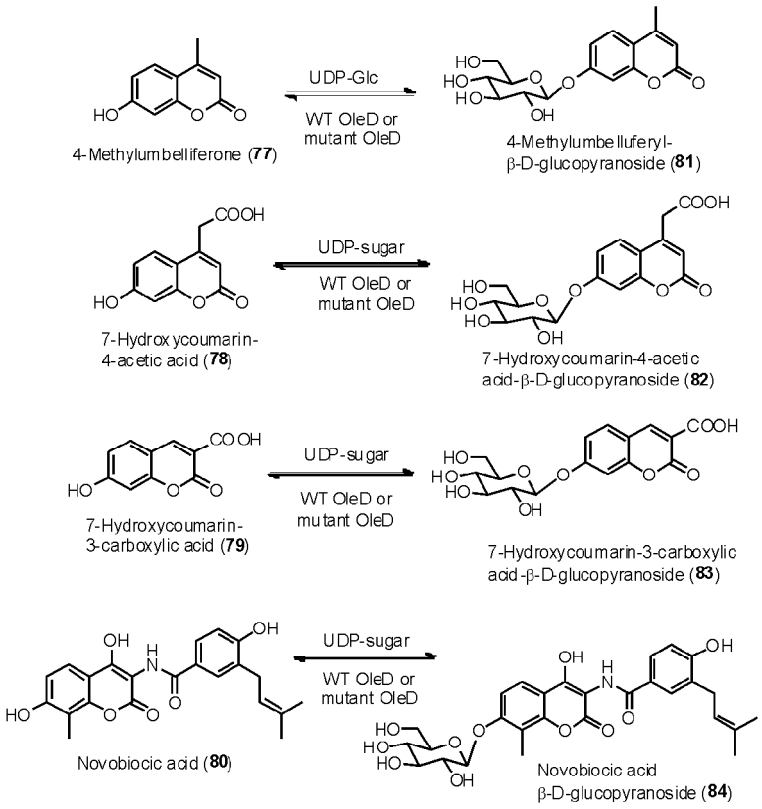
Additional evaluation of single mutant OleD (P67T, I112T, S132F, A242V and G340W) was done with novobiocic acid (**80**) which showed that the first four mutants were able to amplify its glycosylation, where I112T showed the highest activity[82]. Three double mutants (I112T/S132F, I112T/A242V and I112T/P67T) were generated where I112T was invariant substitution and the specific activity of compound **80** and **77** was investigated, which unveiled that incorporation of P67T or A242V (as in double mutant I112T/P67T or I112T/A242V, respectively) could enhance the activity of single mutant I112T. Further incorporation of A242V produced triple mu-



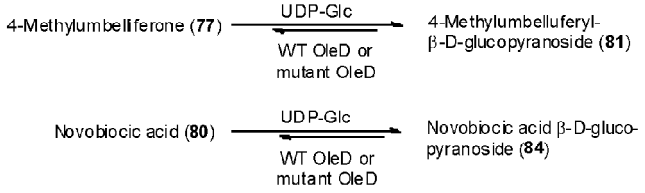
Scheme 7. Diversification of descarbamoylnovobiocin.

tant I112T/P67T/A242V offering an advanced catalyst for glycosylation of novobiocic acid (**80**). Another triple mutant I112T/S132F/A242V was also produced in this series but it was found to be less active than that of the previously mentioned triple mutant (I112T/P67T/A242V). Single-site saturation mutagenesis at Pro67, Ile112 and Ala242 was done in the scaffold P67T/I112T/A242V to generate libraries of 'P67X', 'I112X' and 'A242X', respectively. Library of P67X could not show any improvement in its variants, where several colonies from I112X and A242X showed two to three fold enhancement for glycosylation of compound **80** (Scheme 9)[82]. The substitution of Ala242 with leucine (Leu) was found to be responsible for the activity in the A242X library and two hits from the I112X library possessed lysine. These clones showed higher activity compared to the parent triple mutant P67T/I112T/A242V. Recombination of three finest mutations rapidly identified P67T/I112K/A242V as a variant which is 150 fold active than that of wild-type OleD and 28 fold improved over triple mutant P67T/S132F/A242V[82].

Suspended cultured cells of *Catharanthus roseus* have been found to be able to glycosylate a variety of coumarin moieties for obtaining new coumarin glycosides[83]. Four coumarin aglycons, *i.e.* 7-hydroxy-4-methylcoumarin (**77**), 7-hydroxy-4-phenylcoumarin (**85**), 5,7-dihydroxy-4-methylcoumarin (**86**), 7,8-dihydroxycoumarin (**87**) were used as substrates to get seven coumarin glycosides. Aglycon **77** produced 4-methylumbelliferyl-β-D-glucopyranoside (**81**) and 4-methylcoumarin-7-*O*-β-D-xylopyranosyl(1→6) β-D-glucopyranoside (**88**), where aglycon **85** produced 4-phenylumbelliferone β-D-glycopyranoside (**89**) and 4-phenylcoumarin-7-*O*-β-D-glucopyranosyl(1→6) β-D-glucopyranoside (**90**) (Scheme 10). When aglycon **86** was treated under the same reaction conditions, 4-methylcoumarin-5-*O*-β-D-glucopyranoside (**91**) and 4-methylcoumarin-5,7-*O*-β-D-diglucopyranoside (**92**) were produced (Scheme 10). Aglycon **87** produced 7-hydroxycoumarin-8-*O*-β-D-



Scheme 8. Glycosylation mediated by glycosyltransferases.



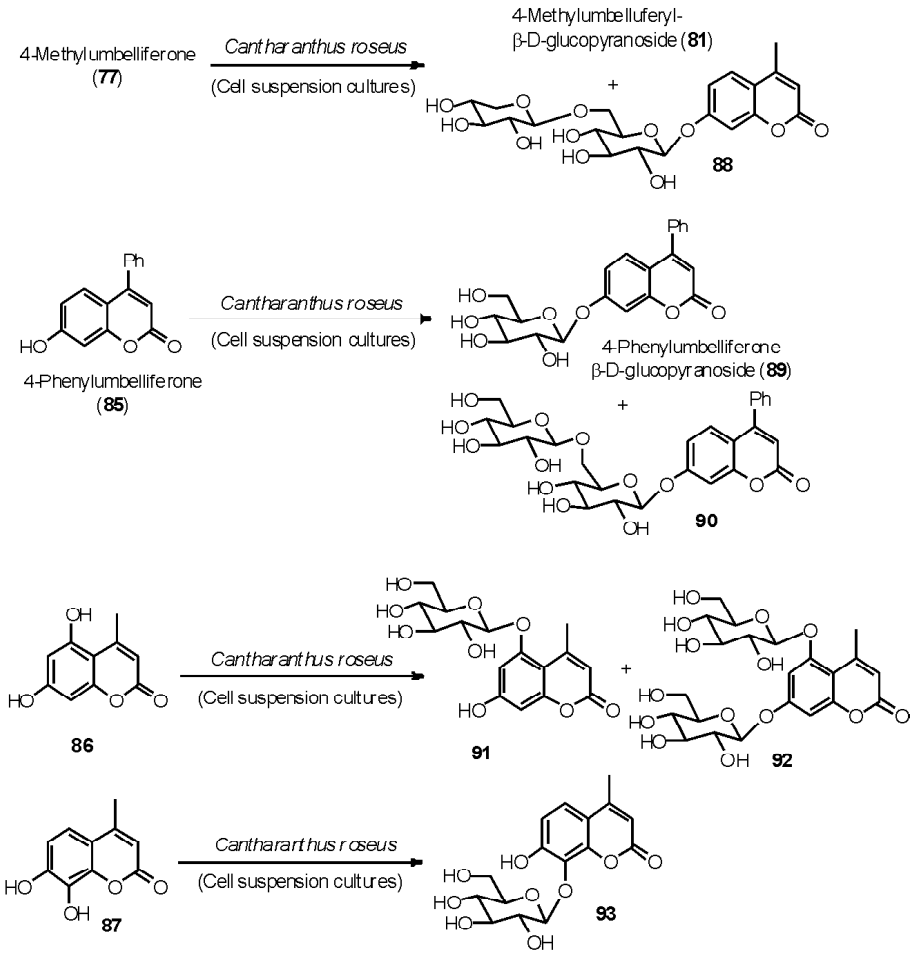
Scheme 9. Novobiocin glycolrandomization by glycosyltransferases mutants.

glucopyranoside (**93**) under same reaction conditions. The cell culture showed its regio-selective nature of glycosylation towards the hydroxyl groups of the exogenous substrates.

Five coumarin aglycons, *i.e.* esculetin (**40**), 4-methylumbelliferone (**77**), scopoletin (**94**), daphnetin (**95**) and hydrangetin (**96**) were glycosylated with UDP-Glc as glycosyl donor by using a glycosyltransferase enzyme, which was isolated and purified from tobacco cell suspension culture[84]. This enzyme showed firm specificity for glycosylation towards the C-7 hydroxyl position in case of both daphnetin (**95**) and esculetin (**40**) to afford coumarin glycosides **99** and **97**, respectively. Scopoletin (**94**) and 4-methylumbelliferone (**77**) were glycosylated by this enzyme to afford coumarin glycosides **98** and **81**, respectively to a lesser extent (Scheme 11).

The crude enzyme preparation from *Daphne odora* was found to hydrolyze daphine (**99**) to daphnetin (**95**), which also produced daphnetin-8-β-D-glycoside (**101**) during the reaction[85]. The reaction mixture developed a bright greenish fluorescence due to the formation of daphnetin (**95**). This enzyme was also found to transfer glycosyl moiety of esculin (esculetin-6-β-D-glucoside, **41**) to daphnetin (**95**) forming coumarin glycoside **101** (Scheme 12). Strict specificity of the enzyme was revealed by further experiments when glucose, maltose, cellobiose and phenolic glycosides were found ineffective as glycosyl donor and umbelliferone (**102**) was not glycosylated by this system.

A phenomenon was reported by Miura *et al.*[86], where 2,4-dichlorophenoxyacetic acid (2,4-D) was found to stimulate the uptake of scopoletin (**94**) from the cell culture medium of tobacco (*Nicotiana tabacum* L. 'Bright Yellow') into



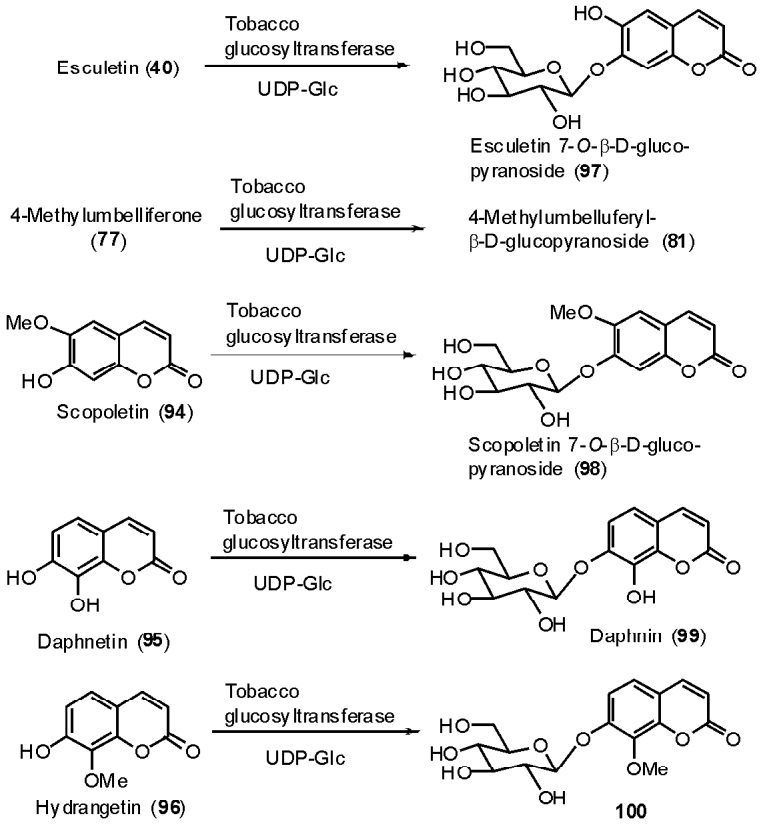
Scheme 10. Glycolysation of coumarin derivatives by cell suspension culture of *Cantharathus roseus*.

the cells and then glycosylating it into its corresponding monoglycoside, scopolin (**98**, Scheme 13)[86]. The plant hormone 2,4-D has been found to stimulate the glucosylation of scopoletin to scopolin by enhancing the UDP-glucose:scopoletin glucosyltransferase (SGTase) activity.

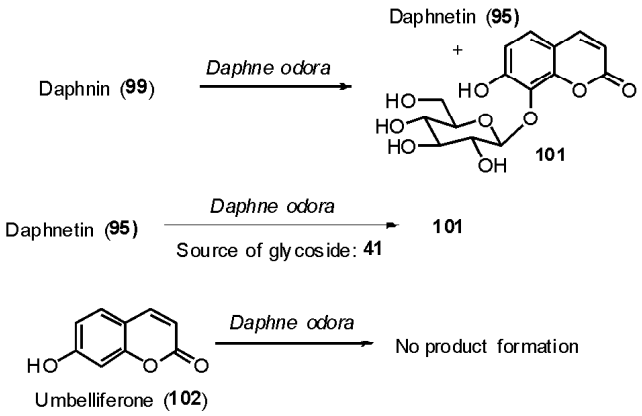
4. Synthesis of coumarin glycosides in hairy root culture system

Hairy roots culture is a kind of plant tissue culture used for studying metabolic processes or for producing secondary metabolites or for biotransformation of substrates in the plant system. This process is also known as transformed root culture. Naturally occurring Gram-negative soil bacterium *Agrobacterium rhizogenes* contains root inducing (Ri) plasmid that infect plant roots to grow unusually and provides a potential plant culture system enabling the high proliferation and biosynthesis of secondary metabolites[87,88]. The development of transformed root systems provides researchers a unique approach to *in vitro* plant biotechnology. The potential of hairy roots for the biosynthesis of natural products was portrayed through a series of experiments focussing on the production of alkaloids[89,90] and coumarin derivatives[91]. During last three decades, several advantages of transformed root culture have been reported, which includes the relative fast growth rates in hormone free medium, biochemical and genetic stability, aptitude for the synthesis of metabolites and scalability of hairy root cultivation[92,93].

Skimmin (**103**) was isolated from the hairy roots of *Pharbitis nil* which showed its ability to produce corresponding aglycon umbelliferone (**102**) as a phytoalexin under stress condition and to store its glycosylated form, coumarin glycoside **103** in the hairy roots as a source of phytoalexin[89]. Fur-

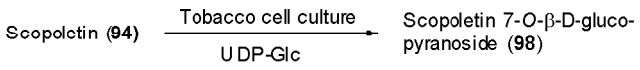


Scheme 11. Glycosylation of coumarin derivatives by glycosyltransferase from tobacco.

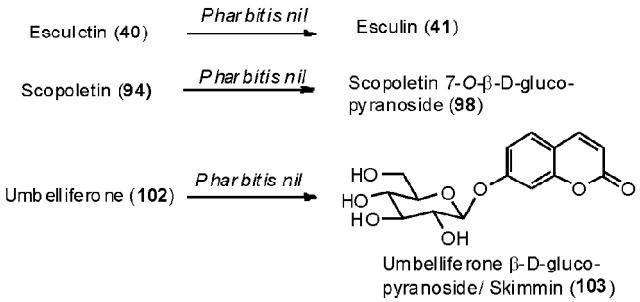


Scheme 12. Conversion of daphnin to daphenetin by crude enzyme from *Daphne odora*.

ther experiments showed that these hairy roots are capable of glycosylating esculetin (**40**) and scopoletin (**94**) to produce coumarin glycosides aesculin (**41**) and scopolin (**98**), respectively (Scheme 14).



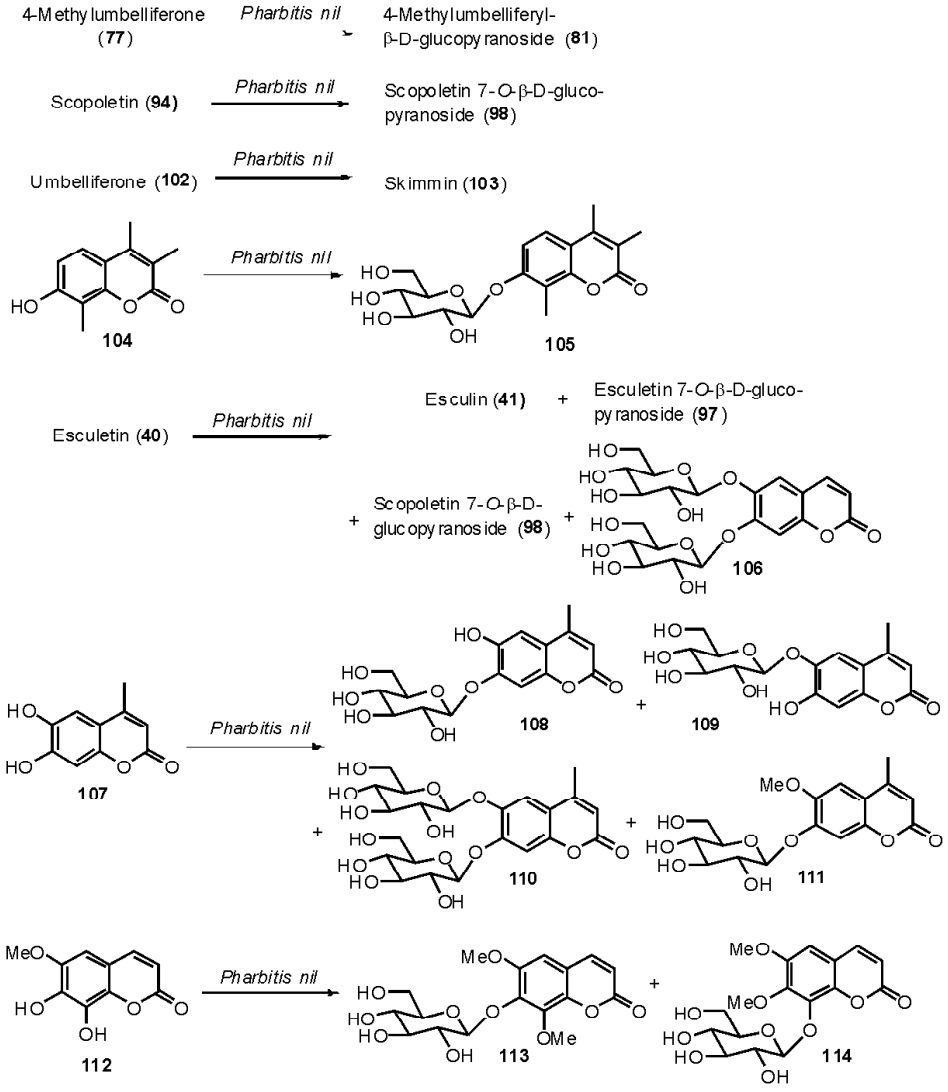
Scheme 13. Glycosylation of scopoletin in tobacco tissue culture.



Scheme 14. Glycosylation of coumarin derivatives by hairy roots of *Pharbitis nil* (I).

Inspired by the potential of hairy roots of *Pharbitis nil* to glycosylate coumarin moieties, Kuroyanagi and co-workers[94]

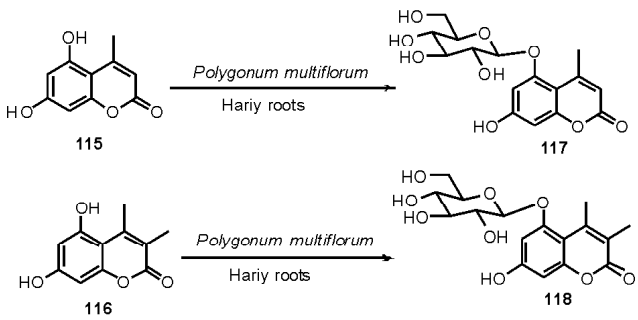
incubated several coumarin aglycons with the hairy roots of *P. nil* to get glycosylated coumarins in twelve hours. 7-Hydroxycoumarin (umbelliferone, **102**) was glycosylated easily to get 7-*O*-β-D-glucopyranosyloxycoumarin (skimmin, umbelliferone β-D-glucopyranoside, **103**) in a very facile way but the introduction of the methyl group at C-4 of the aglycon **77** reduced the rate of glycosylation at the same hydroxyl to get coumarin glycoside **81**. Incubation of scopoletin (6-methoxyumbelliferone, **94**) and 3,4,8-trimethylumbelliferone (**104**) with the hairy roots produced glycosylated products 7-*O*-β-D-glucopyranosyloxy-6-methoxycoumarin (**98**) and 7-*O*-β-D-glucopyranosyloxy-3,4,8-trimethylcoumarin (**105**), respectively in relatively lower yields. When aesculetin (**40**) was incubated with hairy roots, it produced four different coumarin glycosides, *i.e.* 6-*O*-β-D-glucopyranosyloxy-7-hydroxycoumarin (**41**), 7-*O*-β-D-glucopyranosyloxy-6-methoxycoumarin (**98**), 7-*O*-β-D-glucopyranosyloxy-6-hydroxycoumarin (**97**), and 6,7-*O*-β-D-diglucopyranosyloxy-coumarin (**106**) in almost equal amount (Scheme 15). Under the same reaction conditions, 4-methylaesculetin (**107**) produced 7-*O*-β-D-glucopyranosyloxy-6-hydroxy-4-methylcoumarin (**108**), 6-*O*-β-D-glucopyranosyloxy-7-hydroxy-4-methylcoumarin (**109**), 6,7-β-D-diglucopyranosyloxy-4-methyl-coumarin (**110**) and 7-*O*-β-D-glucopyranosyloxy-6-



Scheme 15. Glycosylation of coumarin derivatives by hairy roots of *Pharbitis nil* (II).

methoxy-4-methylcoumarin (**111**) where coumarin glycoside **109** was the major product. Incubation of 6,7,8-trihydroxycoumarin (**112**) with same hairy roots produced monoglucosylated and dimethylated coumarin derivatives, which could be either of compound **113** or **114** (Scheme 15).

Hairy roots of *Polygonum multiflorum* were found to glycosylate two coumarin moieties (**115** and **116**) when these aglycons were suspended with the culture medium of *P. multiflorum* and shaken for days[95]. The biosynthesized compounds were identified as 7-hydroxy-4-methylcoumarin 5-*O*-β-D-glucopyranoside (**117**) and 7-hydroxy-3,4-dimethylcoumarin 5-*O*-β-D-glucopyranoside (**118**, Scheme 16), respectively on the basis of chemical and spectroscopic analysis.

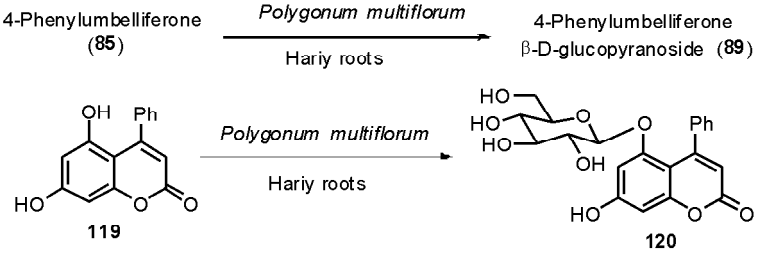


Scheme 16. Glycosylation of coumarin derivatives by hairy roots of *Polygonum multiflorum*.

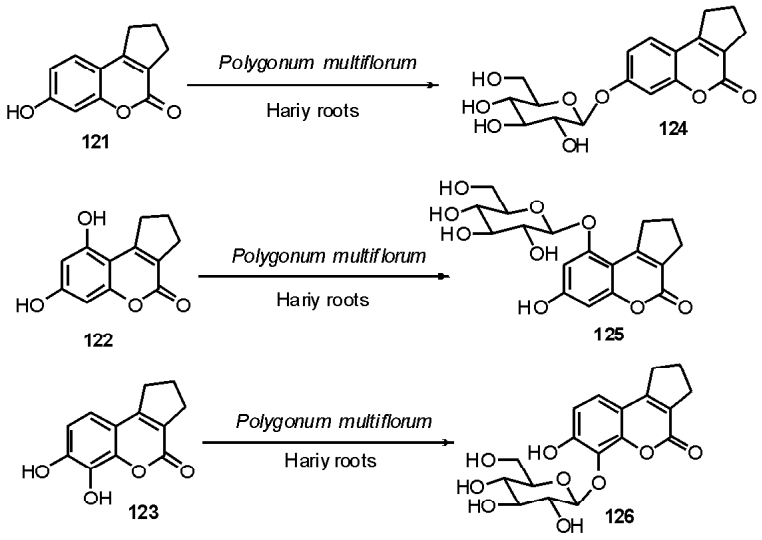
A chemoenzymatic synthetic method was developed for the preparation of coumarin glycosides when chemically synthesized 7-hydroxy-4-phenylcoumarin (4-phenylumbelliferone, **85**) and 5,7-dihydroxy-4-phenylcoumarin (**119**) were glycosylated by transgenic hairy roots of *Polygonum multiflorum* to obtain two biosynthesized coumarin glycosides, 4-phenylcoumarin-7-*O*-β-D- glucopyranoside (**89**) and 7-hydroxy-4-phenylcoumarin-5-*O*-β-D-glucopyranoside (**120**), respectively (Scheme 17)[96].

Three coumarin glycosides 7-*O*-β-D-glucopyranosyl-2,3-dihydrocyclopenta[*c*]chromen-4-one (**124**), 9-*O*-β-D-glucopyranosyl-7-hydroxy-2,3-dihydrocyclopenta[*c*]chromen-4-one (**125**) and 6-*O*-β-D-glucopyranosyl-7-hydroxy-2,3-dihydrocyclopenta[*c*]chromen-4-one (**126**) were biosynthesized using transgenic hairy roots of *Polygonum multiflorum*[97]. Generation of these three 3,4-cyclocondensed coumarin glycosides **124**, **125** and **126** from their corresponding aglycon moieties, i.e. compounds **121**, **122** and **123**, respectively showed the regio-selective nature of the hairy roots (Scheme 18).

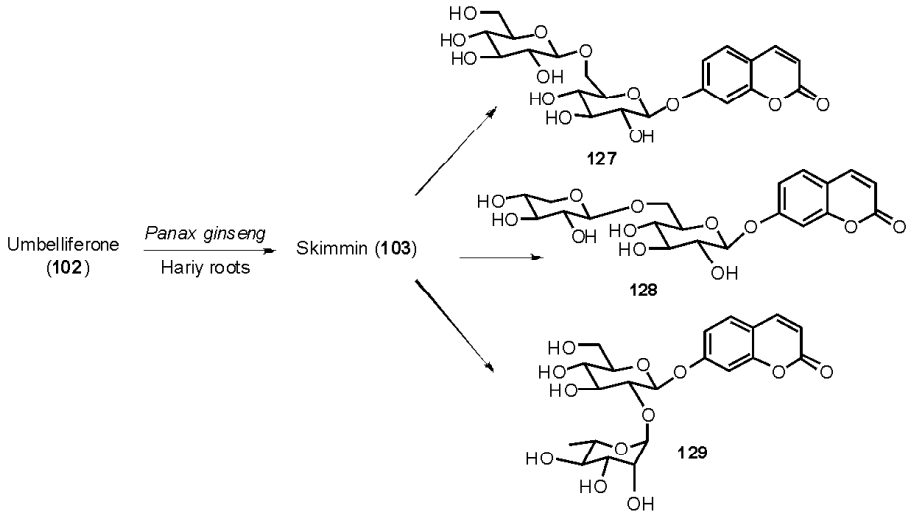
Biotransformation of umbelliferone (**102**) was carried out using *Panax ginseng* root cultures, which showed the high capability to glycosylate the 7-hydroxycoumarin molecule[98]. Methanolic extract of the roots provided a crude product, which was further separated to obtain one monoglycosylated coumarin and three diglycosylated coumarins (Scheme 19). Monoglycosylated coumarin was identified as 7-*O*-β-D-glucopyranosyl-umbelliferone (Skimmin, **103**) and three diglycosylated coumarins were identified as umbelliferone 7-*O*-β-D-glucopyranosyl(1→6) β-D-glucopyranoside (**127**), umbelliferone 7-*O*-β-D-xylopyranosyl(1→6) β-D-glucopyranoside (**128**) and umbelliferone 7-*O*-α-L-rhamnosyl (1→2) β-D-glucopyranoside (**129**).



Scheme 17. Glycosylation of coumarin derivatives by transgenic hairy roots of *Polygonum multiflorum*.



Scheme 18. Glycosylation of fused-coumarin derivatives by transgenic hairy roots of *Polygonum multiflorum*.



Scheme 19. Glycosylation of umbelliferone (**102**) and skimmin (**103**) by *Panax ginseng* root cultures.

5. Summary

Among thousands of different naturally occurring compounds, coumarins constitute a class of compounds that have immense biological significance. In this review, we have focussed on the biochemical approaches, i.e. the mutagenic synthetic processes, chemoenzymatic processes and hairy root culture systems for the synthesis of coumarin glycosides. In past decades, the utilization of coumarin glycosides in the medicinal chemistry field and other applied fields has increased many folds. Accordingly, the demand for exploration of newer coumarin glycosides is also high. This review will let the researcher have an inside view and a clear perspective towards the available synthetic methodologies for biochemical preparation of coumarin glycosides. This review will also serve the purpose of a databank of biochemically synthesized coumarin glycosides.

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