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Hexenoses in design of glycoconjugates - from chemistry to function

Grzegorz Grynkiewicz*[1], Wieslaw Szeja[2], Piotr Krzeczyński[1], Aleksandra Rusin[3]

[1]*Pharmaceutical Research Institute, Warsaw, Poland*
[2]*Chemistry Department, Silesian Technical University, Gliwice, Poland*
[3]*M. Sklodowska-Curie Institute of Oncology, Gliwice, Poland*
**Email: G. Grynkiewicz; g.grynkiewicz@ifarm.eu*
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(This paper is dedicated to the memory of late Dr. Jan Ramza [1958–2013], an enthusiastic synthetic chemist, whose significant and useful contribution to the field of carbohydrate chemistry deserves lasting remembrance).

Abstract: Reaction of pyranoid glycals with various nucleophiles, known as Ferrier rearrangement, which leads to hex–2–enose derivatives is generally perceived as an useful synthetic transformation, but its scope regarding complex substrates, such as secondary metabolites abundant in plants (e.g. polyphenols), is not fully recognized. Although no specific glycosyltransferases (GTS) for unsaturated pyranoses are known and consequently, hexenoses are rare in Nature, an interest in their application as synthetic scaffolds is growing constantly. Thus, total synthetic approaches, like Achmatowicz rearrangement, which utilizes simple furan carbinols, have matured to the level of fully stereocontrolled transformation, providing viable alternative for using natural sugar synthons. Taking up isoflavones as exemplary phenolic secondary metabolites of considerable interest for medicinal chemistry, their conjugation leading to hex–2–enose mimics of natural glycosides is discussed in this review from the point of view of chemical synthesis, principally from glycal derivatives, as well as regarding biological activity of newly obtained compounds.

Keywords: hex-2-enopyranoses; Ferrier rearrangement; isoflavone glycoconjugates; genistein glycosides; biological activity of genistein derivatives.

1. Introduction

Relatively recently, basic concepts of glycobiology concerning mainly glycoproteins, polysaccharides and glycolipids [1] [2] [3] started to merge with synthetic chemistry of oligosaccharides and low molecular weight glycoconjugates, perceived as a subject of

medicinal chemistry and drug discovery area [4] [5] [6], reflecting growing interest in sugar moieties as an information–rich, property modulating building blocks. In line with this general trend, our continuous interest in glycosides of natural origin and their semi-synthetic analogs develops in accord with generally accepted idea of natural products pool as a superb quality mega library of biocompatible and bioactive compounds of great interest to experimental life sciences, as well as to the theory of bioinformatics and experimental medicinal chemistry. Among numerous categories of natural glycosides with pronounced biological activity which already have found application in medicinal practice there are clearly two different classes:

i. In the first group, member glycosidic structures are indivisible from the point of view of their biological activity. Most of carbohydrate containing antibiotics (aminoglycosides, anthracyclines, macrolides, nucleoside antagonists, etc) belong to this category. De-glycosylation of such metabolite usually inactivates the compound.

ii. In the second group, glycosides and corresponding aglycones are often present in biological matrix side by side, their activities can be comparable or distinctly different, but presence of sugar is not critical factor for biological action. Large classes of natural products: e.g. flavonoids and saponins, abundant in the Plant Kingdom serve as a good example to illustrate the point.

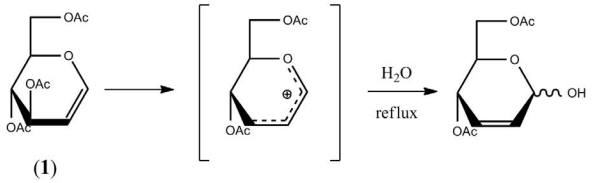
Both categories inspire considerable efforts in design and synthesis of conjugates mimicking natural glycosides by sugar moiety structure modification and this review also concerns this particular topic, focusing narrowly on hex–2–enosides of flavonoids. Interestingly, among nearly hundred families of glycosyltransferases (GTs), there have not been found enzymes capable of transferring unsaturated pyranosides, hence there are no natural hex–2–ene pyranosides [7] [8] [9] (neuraminic acid derivatives constitute separate class, as heptose derived family with 1–carboxyl group facilitating formation of conjugated double bond which in graphical structural representation resemble glycals, notwithstanding, they played crucial role in discovery of glycal umpolung induced by samarium diodide [10]). Consequently, the topic announced in the title of this article might be considered as being artificial and impractical. On the other hand, arguments to the contrary could be brought forward easily. It is known from examples, that natural sugar moieties of native glycosides and glycoconjugates can be modified by synthetic means (or biotransformation), with beneficial results for such physicochemical features, which govern most important characteristics of a prospective glycosidic drug candidate along ADMET axis, improving it overall efficiency. Moreover, following these observations, purely artificial glycoconjugates and glycomimetics have been designed and introduced in biological activity testing with fair deal of success. Therefore, it can be postulated that carbohydrate moieties (and oligosaccharide assemblies), no matter how advanced in structural modification, can be useful for a prospective conjugative modification of currently interested pharmacophores undergoing drug discovery and development process. By such a token, hex–2–enopyranoses have already been validated as carriers of useful biological properties, as demonstrated in several examples discussed further down in the final paragraphs of this review.

As illustrated in contemporary monographs [11] [12] in both groups of glycosides listed above, exploratory chemical glycosylation of the native aglycones has done valuable service to accumulation of useful knowledge, and affording new compounds in which for

example natural antibiotic sugars were replaced with modified glycosyl units, often generating compounds with considerably improved pharmacokinetic and pharmacological properties. Among typical textbook chemical glycosylation methods, the use of acylated glycals for synthesis of 2,3–unsaturated 1–*O*–pyranosides, stands out as a protocol using stable, easily accessible reagents and simple, user friendly procedures. The products — hex–2–enose glycosides, are distinctly different from those obtained in classical Koenigs–Knorr or Schmidt protocols [13] [14], nevertheless effects of such glycosylation in terms of biological activity of novel conjugates are noteworthy [7] [15]. In our opinion, isoflavone derivatives which contain hex–2–enopyranose moiety, obtained and studied during the last decade or so, represent new quality, at least in a search for anticancer drug leads [16] [17]. Therefore, chemical synthetic methods, leading to regioselective and stereoselective placement of such moiety within phenolic natural products (e.g. these with flavonoid framework) deserve some critical evaluation.

2. Overview of synthetic methods for preparation and chemical transfer of hex–2–enopyranose moiety by Ferrier rearrangement and related transformations

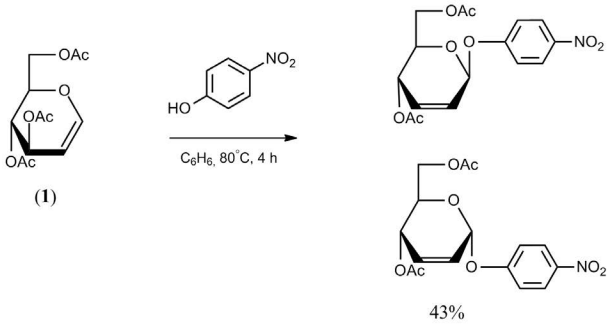
As Robin J. Ferrier himself remarked in the comprehensive review on the topic [18], the first case of substitutive rearrangement of acetylated glycal (**1**) to hex–2–enopyranose was observed by Emil Fisher when he, a century ago, applied hydrolytic conditions to the product of reductive elimination of peracylated D–glucosyl bromide



[19] (Scheme 1).

Scheme 1

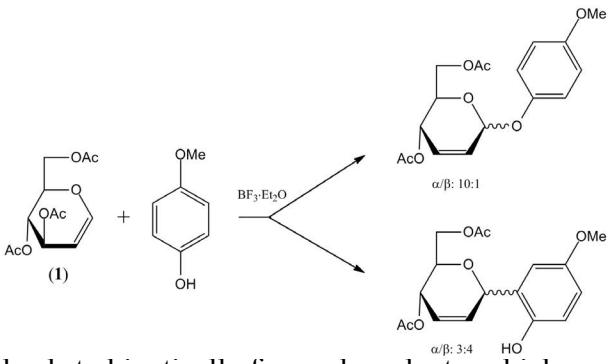
Half a century later made observation on thermal



transformation of (**1**) in the presence of 4–nitrophenol (Scheme 2) [20] opened a pathway, which looked appealing from synthetic point of view.

Scheme 2

It soon turned out that this transformation is quite general and in fact it has started a new evergreen method of chemical glycosylation soon named "Ferrier reaction" (FR shortening will be used further occasionally throughout the review). It formally belongs to the category of allylic substitution classified as S_N2' transformation [21]. Since that time many kinds of glycals rearrangement with concomitant nucleophilic displacement have been described, in which hex–2–enoses are formed under great variety of conditions, and apparently by different mechanisms [22] [23] [24]. It should be noted, that in case of electron rich phenols as substrates, two different chemoselectivities can be observed under FR conditions. Thus, 4–methoxyphenol reacted with 3,4,6–tri–*O*–acetyl–D–glucal (**1**) in toluene in the presence of catalytic amount of BF_3 etherate affords an anomeric mixture of *O*–glycosides, while the same substrates reacted in DCM as solvent give exclusively *C*–linked hex–2–enoses (Scheme 3) [25]. It has been reasoned that this reaction represents a two-step process: *O*–glycosylation



leads to kinetically favored products, which can be further rearranged to thermodynamically favoured *C*–conjugates.

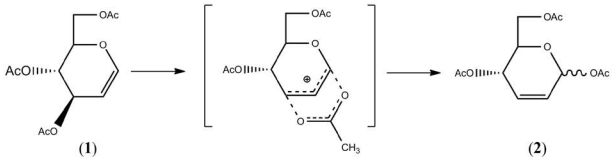
Scheme 3

Nucleophilic substitution in simple allylic system belongs to the most important transformations of synthetic chemistry, by which C–C and C–heteroatom bonds are created. Theory of this particular reaction has been discussed in considerable detail in number of monographs. It considers properties of the leaving group, various catalysts and incoming nucleophile, often in terms of molecular orbital theory. Nature of the metal catalyst complexes with π–allylic electron system (as well as possible σ bonding) was thoroughly studied and well documented in numerous cases. Matter of stereoselectivity in outcome of S_N2' substitution reactions is also of particular concern in general discussion of the transformation [21].

However, in the case of unsaturated pyranosides, the matter seems to be much more empirical and to our knowledge no theory has been advanced to explain multidirectional reactivity of glycals in the presence of various catalysts. In comparison with simple C_3 π–allyl system, there are additional factors to account for, like electronic participation of the ring oxygen, which introduces enough reactivity difference to support argument for a separate category (thus the name reaction — known as Ferrier rearrangement [26], FR). In general, glycals are perceived as electrophilic synthons, which are able to generate bidentate oxocarbonium ions, through complexation and heterolytic bond dissociation of C–3 substituent in form of an anion. Although acetylated glycals are routinely applied in FR reactions, some attention has been paid to reactivity of unprotected glycals [27] [28] [29] [30] and their C–3 substituted derivatives with enhanced leaving group properties [31] [32]. An intermediate, delocalized oxocarbonium ions, formed by dissociation of suitably complexed C–3 leaving group, readily react with variety of nucleophiles, with formation of new C–O, C–N, C–S and C–C bonds, often affording C–1 substituted hex–2–enoses as the main products. Although this reaction has wide scope; it has been successfully applied in numerous syntheses of natural carbohydrate derivatives [18] and their mimetics with potential biological activity [33]. There are also some considerable limitations. For example complex multifunctional nucleophilic substrates perform poorly in reactions with glycals, while natural products classified as polyphenolics seem rather unreactive or undergo side reactions only. Additionally, there is no general guideline concerning choice of catalyst for a reaction without prior experimental record. Initially, there has been a rule of thumb, indicating that dual reactivity of cyclic vinyl ethers: 1,2–addition and S_N2' — rearrangement could be controlled by application of a catalyst — Brønsted (protic) acid favouring addition, while Lewis acids promoted the double bond shift — both transformations prone to kinetic, as well as thermodynamic control which might operate under particular conditions. Unfortunately, in view of experimental facts collected from recent literature, there is not enough support for a simple and clear cut division presented above; for example phosphoric acid was found to be a selective and effective catalyst for rearrangement [34] [35] [36] [37] as well as $HClO_4$ (protic) supported on silica gel [38], while conversely — 1,2 additions have been

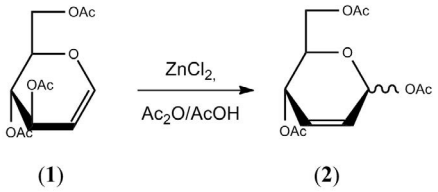
observed during Lewis acid catalysis [39]. Thus, a quest for new applications of the FR, in particular in the field of complex natural products, requires recapitulation and critical appraisal of well documented examples from current literature.

In principle, acylated glycals, like the most popular member of the class, commercially available 3,4,6–tri–*O*–acetyl–D–glucal (**1**) should be susceptible to 1,3 sigmatropic rearrangement (thermal) (Scheme 4).



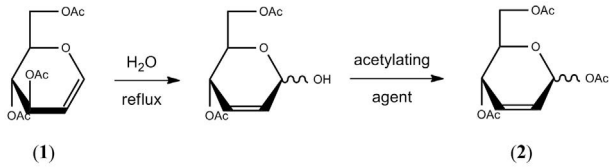
Scheme 4

Although early works of British researchers presented evidence for such transformation, its practical utility remained insignificant. Availability of 1–*O*–acyl–2,3–unsaturated hexenoses has been addressed by V. Sunjič, who studied reaction of (**1**) in AcOH/Ac_2O mixture catalysed by zinc chloride (Scheme 5). This reaction affords expected hexenose product, but is accompanied by the glucal dimerization [40].



Scheme 5

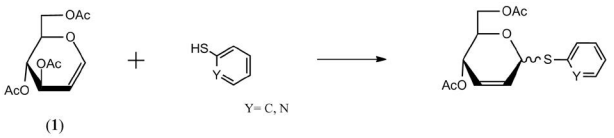
It seems that more practical way to the anomeric mixture of pseudoglycals is a two-step procedure: hydrolysis followed by acetylation (Scheme 6).



Scheme 6

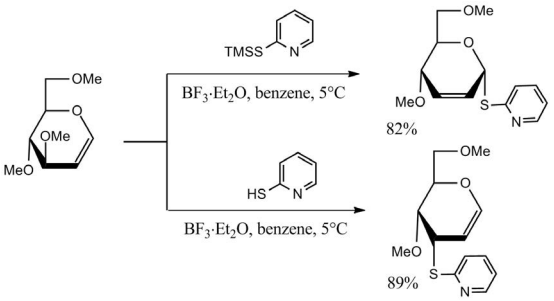
Obviously, the issue of interconversion of both unsaturated compounds is complicated by question of anomeric configuration and stereochemistry of the acyl transfer. Although it has been generally agreed that catalysed allylic rearrangement of glycals (FR) should proceed through a common intermediate — 1,3–delocalized carboxonium cation, we are not aware of a study which would juxtapose reactivity towards external nucleophiles, paricularly in view of possible significance of kinetic effects. Much of an useful empirical knowledge about FR has been accumulated from great abundance of papers describing reactions of glycals with *O*–, *S*–, *N*– and *C*– nucleophiles [18] [22] [34] [35] [36] [41]. Since *O*–glycosylation reactions using FR protocols have been reviewed extensively and repeatedly we would only like to alert the readers that enormous variety of condition tried (e.g. various kinds of catalysts) generated plethora of results, with sometimes inconsistent claims, which makes it difficult to make general recommendation for new preparations. In general, it looks like success in direct FR type glycosylations with complex and multifunctional phenolic aglycons, in contrast to alcohols, are rather unlikely. For comparison, some reactions of glycals with heteroaromatic are briefly presented and discussed below. Discussion of C–H nucleophiles with glycals follows as a short separate paragraph.

Generaly, glycals like (**1**) react with tiophenols and their heterocyclic analogs leading to *S*–gycoconiugates (Scheme 7):



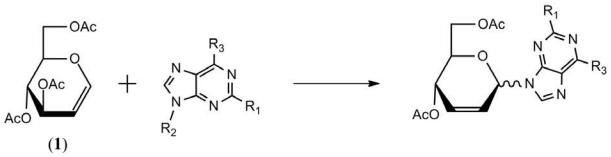
Scheme 7

Regioselectivity of theese reactions may depend on various conditions e.g. presence of protective group (Scheme 8):



Scheme 8

Reactions of glycals with biologically important heterocyclic amines like protected adenine or chloropurine are well known (Scheme 9) and lead to appropriate *N*–glycosides:



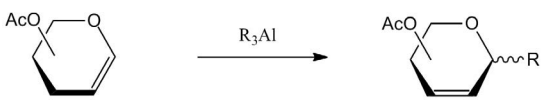
Scheme 9

Literature describes also many examples of FR of cyclic imides and suitably protected: uracils, cytosines, theophyllines and 1*H*–bezotriazoles. Since our main concern is the utility of the Ferrier rearrangement for functionalization of complex phenols of natural origin, *O*– and to lesser extend *C*– FR are of primary importance and only a brief critical overview of other substrate reactions is presented.

3. Reactions of glycals with electron-rich carbon substrates leading to C–glycosidic hexenoses

Among wide selection of synthetic methods employed for synthesis of *C*–glycosides, these involving glycals were reviewed periodically [42] [43] [44] providing panoramic view of new developments in transformations, frequently described as carbon–Ferrier rearrangement. These new developments often involve new, sophisticated methods of substrate activation (e.g. MW assistance [45]), like rather exotic catalysts [41] and unusual solvents [46] [47]. Generally, acylated glycals are recognized as ambident electrophiles, rather easily activated in the presence of Lewis acids, which assist transformation of 3–*O*–ester group into a good leaving group. Historically, classical electrophilic aromatic substitution [48] [49] preceded a study on FR with cyanide anion (and later TMSCN) as a nucleophile, which represents an example of synthesis hex–2–enoses with reactive C_1 anomeric substituent [50] [51]. The scope of this reaction quickly expanded by application of such electron–rich substrates as enol esters, phenol derivatives and five membered ring heterocycles [48] [49] [50] [52].

Since *C*–glycosidic bond synthesis is of constant interest, as a viable entry to a specific category of natural flavonoids [53] and also to metabolically stable congeners of various natural *O*–glycosides, which can serve as enzyme inhibitors, great variety of other variants have been elaborated [54] [55] [56]. List of successfully applied substrate categories include: allyl silanes, propargyl or alkynyl silanes, allene derivatives, enol silyl ethers, esters and sulfonates, isocyanides, olefins, organometalic nucleophiles, organoboron compounds, etc. [44]. Representative examples of FR type *C*–glycosylations, from rich literature of the subject, are shown on the following schemes. Examples start with not so obvious reaction of acetylated–D–glycals with trialkylaluminum reagents [57] in which direct ligand exchange seems to take place between metal and sugar derived oxocarbonium intermediate (Scheme 10). Stereoselectivity of this reaction is remarkably dependent upon Lewis acid catalysts, which are selected from lanthanide triflates [41] [58].

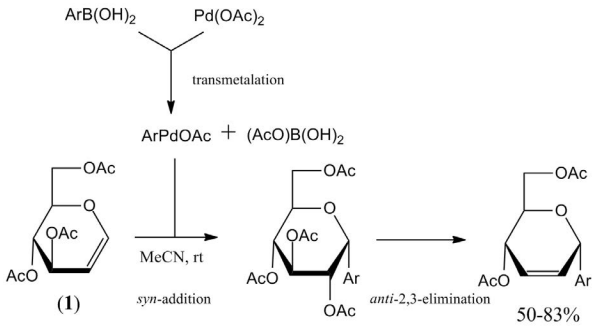


Scheme 10

Analogously, *C*–aryl glycopyranosides can be obtained in the reaction with triarylindium reagent [59]. Reaction undergoes via indium(III) Lewis acid assisted ionization and involves formation of an oxocarbonium ion.

Aryl Grignard reagents and simple dialkylzinc compounds perform similarly [60], but complex organozinc derivatives (Reformatsky reagents) have found much wider application because of good tolerance for various functional groups and very good yields [61] [62] of *C*–glycosidic products.

In case of palladium–catalysed reactions leading to hex–2–enose *C*–glycosidic products, which starts from popular arylboronic acids as substrates, there are three distinct steps of the bonds rearrangement: transmetalation, *syn*–addition of an aryl–palladium compound to the glycal double bond, and *anti*– 2,3–elimination (Scheme 11), with possible 2,1–elimination as a competing process.



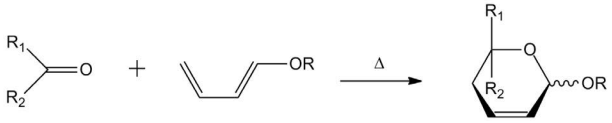
Scheme 11

Synthesis of allylic aryl ethers is to be done via palladium(0) catalyzed arylation of allylic carbonates, [63] [64].

C–Silylated electron–rich compounds (eg. alkyl, allyl, allenyl, alkynyl) are by far the most popular substrates for FR, aimed at formation of an anomeric carbon–carbon bonds. Thus, allyltrimethylsilane reacts readily with variety of glycals, in the presence of equimolar amount of titanium tetrachloride as a Lewis acid catalyst, affording excellent yields of 1–*C*–allyl–hex–2–enoses, with good α selectivity [44]. 1–*C*–pyranosyl acetylenic derivatives can be obtained by analogous transformations.

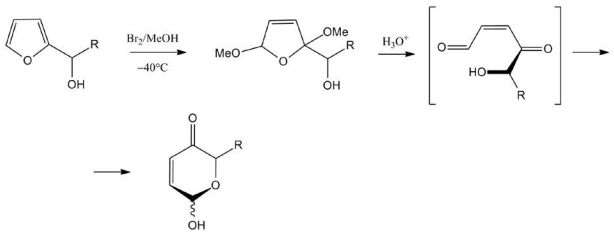
4. Hexenoses as synthetic intermediates and glycosyl donors

There are many approaches to synthesis of saccharides, which stem from achiral synthons and employ unsaturation along the synthetic pathways [65]. Among these total synthetic efforts at least two deserve to be mentioned here, because of clear resemblance of their principal intermediates to the hexenoses obtainable via the Ferrier rearrangement reactions discussed above. Hetero Diels–Alder cycloaddition, when performed on 1–alkoxy dienes with carbonyl dienophiles (e.g.: formaldehyde, carbonyl cyanide, glyoxalate or mesoxalate esters) leads in a regioselective process to dihydropyran derivatives, which can be made up into regular or modified hexoses, but attempts to induce significant enantio– or diastereoselection in these reactions with help of chiral auxiliaries met with only moderate success [66] (Scheme 12).



Scheme 12

Another approach is based on 1,4–oxidative transformation of furan nucleus, which leads to protected maleic aldehyde derivatives. In case when 2–furylcarbinols are used as substrates, acid-catalysed rearrangement, transforming intermediate five–membered oxygen ring into hex–2–ene–4–ulose takes place. This reaction, first described in 1971 [81] is known in contemporary literature as Achmatowicz rearrangement, and it is also practiced in aza– version for syntheses of variety of *N*–heterocycles [67][68] (Scheme 13).

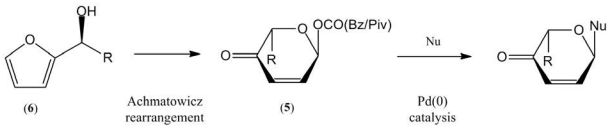


Scheme 13

Utility of the above transformation for preparation of racemic monosaccharides is obvious and has been thoroughly exploited [69] [70]. At the time of discovery, demonstration of its applicability to synthesis of enantiomerically pure sugars required considerable additional effort, e.g. for separation of 1–(2–furyl) ethanol racemate [71] [72]. Presently, such starting materials are readily available from the corresponding ketones, by reduction in the presence of Noyori type catalysts [73]. Therefore, furyl alcohols are often used in multistep reactions leading to synthesis of complex natural products, as synthons equivalent to hex–2–ene–4–ulose moiety, which may be functionalized into variety of hexoses and also enjoys good reputation as a reactive glycosylating reagent [73] [74].

It is obvious that double bond functionality of a hexenose can be further transformed in variety of standard reactions like hydroxylation, epoxidation, hydroxyamination, etc. [75]. Recently, also free radical thiol addition has been successfully exploited for derivatization of hexenoses [76]. Observed preponderance of C–2 axial addition of thiols suggests considerable synthetic potential of this approach.

2,3–Unsaturated *C*–glycoside scaffolds have been named as highly functionalized materials containing three stereogenic centers, easily accessible in large laboratory scale as orthogonally protected synthons, for multidirectional couplings and transformations [77]. Needless to say, variety of 2,3–unsaturated pyranosides with a potential leaving group at the anomeric position, can also serve as glycosyl donors for synthesis of conjugates containing more complex aglycones [78] [79]. Particularly fruitful developments along this line, with excellent control of the anomeric stereoselectivity were achieved by application of hex–2–ene–4–uloses (**5**), under Pd(0) catalysis [80]. Unsaturated synthons for such transformations are readily available by Achmatowicz rearrangement [81] of furfuryl alcohols (**6**) (Scheme 14). A non–catalysed reaction of *O*–nucleophiles with glycal allyl epoxides, which also leads to hex–2–enoses is of special interest because of high degree of stereocontrol induced by oxirane ring stereochemistry [23] [84].



Scheme 14

Although in this review we have concentrated our attention on reactivity of substituted 1,2– and 2,3–hexenoses perceived as a source of bidentate oxocarbonium ions, thus species susceptible for nucleophilic substitution. It should be remembered that umpolung of this reactivity is possible, as demonstrated recently by French researches [10] with help of samarium diodide as the catalyst.

5. Chemical glycosylation of isoflavones

As we have already pointed out, within large group of natural products composed of phenolic glycosides, no hexenose type have been found thus far. Therefore, a subject of our review stands in a special context, based on a delicate balance between natural products and their mimics, usually obtained by means of chemical synthesis. In a golden period of natural product chemistry, marked with introduction of spectral methods of structural analysis in the second half of XX century, great many secondary metabolites have been classified as flavonoid

glycosides, and two distinct categories: *O*–glycosidic and *C*–glycosidic were recognized, based on susceptibility towards enzymatic and chemical hydrolysis. At that time, there was a customary academic obligation to present a final proof of a newly elucidated natural product structure, in form of chemical synthesis of the identical entity. Limited synthetic resources available in the period for chemical glycosylation concentrated on *O*–couplings of simple sugar donors with phenolic natural products, providing great many proof of structures sought. Gradually, researchers in the field adapted some modern synthetic solutions as chloroimidate anomeric leaving group or phase transfer catalysis conditions for acetohalogenose type glycosylating synthons [14] [83]. Nevertheless, aromatic *O*–glycosylation remain to be perceived as a separate category of synthetic transformation for both — mechanistic [84] and methodological [12] [85] reasons.

At the present time, incentive of chemical glycosylation efforts are somewhat different, striving for novel structures which often mimic natural products, with considerable degree of freedom in structural design. In one of early projects of genistein derivatization, FR was attempted with several various glycal derivatives, all in vain. It took considerable concentrated effort to adopt known synthetic transformation for conjugating isoflavones with unsaturated sugar moieties.

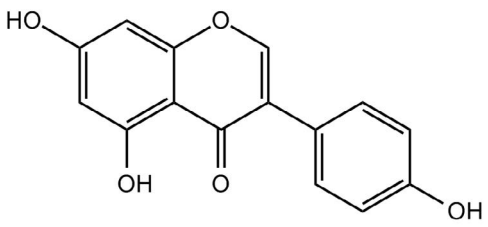


Figure 1

Genistein (Fig. 1), one of the most extensively studied isoflavones, is generally recognized as a phytoestrogen, since the time of discovery that its presence in plant material used as fodder can cause reproductive problems in domestic and captive animals [86] [87]. Meanwhile, isoflavones in general and genistein in particular have been identified as ligands of many more macromolecules than just beta estrogen receptor and have been indicated as modulators of many physiological and pathological processes vital for human health [88] [89] [90]. These findings, reported in numerous monographs scattered over chemical, nutrition, biochemical and medicinal literature have been also recently summarized in a couple of books [91] [92].

Although many studies indicated that presence of a substituent in C7 or C4' position eliminates estrogenic properties of genistein, the glycosidic moiety in native secondary metabolites may play a role in temporary protection and improvement of its oral bioavailability [93]. Bioavailability of pure isoflavones (and their glycosides) in healthy humans and analysis of commercial soy isoflavone supplements were determined with help of HPLC analysis [94] [95] [96]. However, values obtained for plasma concentration were considerably lower than these achieved in cell lines and animal pharmacology experiments. Thus, one can speculate that certain genistein derivatives, even though substituted at the sites critical for estrogenic properties of genisten molecule, will pass the gastrointestinal tract, and after metabolic detachment of the glycosidic carrier, deliver genistein to the site of its action. This hypothesis is currently the subject of *in vivo* bioavailability studies of different genistein glycosides and glycoconjugates.

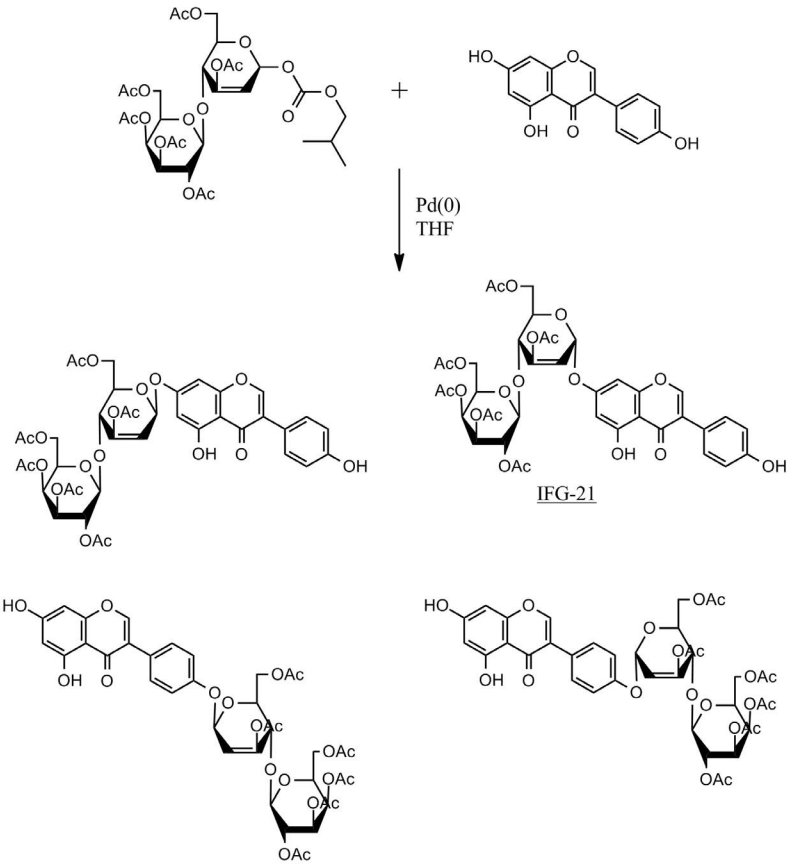
Another discovery that rendered genistein important position as a molecular probe were the findings which demonstrated the correlation between its cytotoxic and antiproliferative activity and inhibition of tyrosine kinases [97] [98] [99] and inhibition of topoisomerase II [100], as well as induction of G2/M block of the cell cycle [101]. These mechanisms of action attracted attention of researchers to potential

use of genistein as a lead in designing anticancer agents.

Glycosylation experiments with genistein were, as in case of other flavonoids, performed principally with application of acylated halogenoses. First synthesis of genistin, amounting to only 17% yield, was reported by Zemplen and Farkas [102]. Wähälä and her co–workers, have managed to obtain yields in range of 40 % by applying phase–transfer catalysis method for genistin glycosylation [103] [104]. More recently, the subject of chemical glycosylation of flavonoids was taken up by several research groups [105] [106] [107]. The latter paper describes high yielding glycosylation of isoflavones. Several authors concluded that efficient 7–*O*–glycosylation requires protection of 4'–OH group. 2,2,2–Trifluoro–*N*–(*p*–methoxyphenyl) acetamidates were used as efficient glycosyl donors and n–hexanoyl groups were employed for temporary protection of phenolic functions [12] [108]. General reactivity of genistein, including glycosylation attempts, was summarized in a recent review [109].

6. Novel developments in synthesis of the isoflavone-hexenose glycoconjugates

To our knowledge, there is no report in literature of successful direct Ferrier type glycosylation, involving a glycal and a polyphenolic substrate from flavonoid category and our own experience with such reactions was also negative, despite of wide variety of conditions tried. It has turned out, however, that in case of isoflavones, desired unsaturated glycosoides could be obtained from pre-formed hex–2–enose donors, under palladium catalysed anomeric exchange, according to published procedures [63] [64]. Thus, genistein, treated with unsaturated 1–*O*–

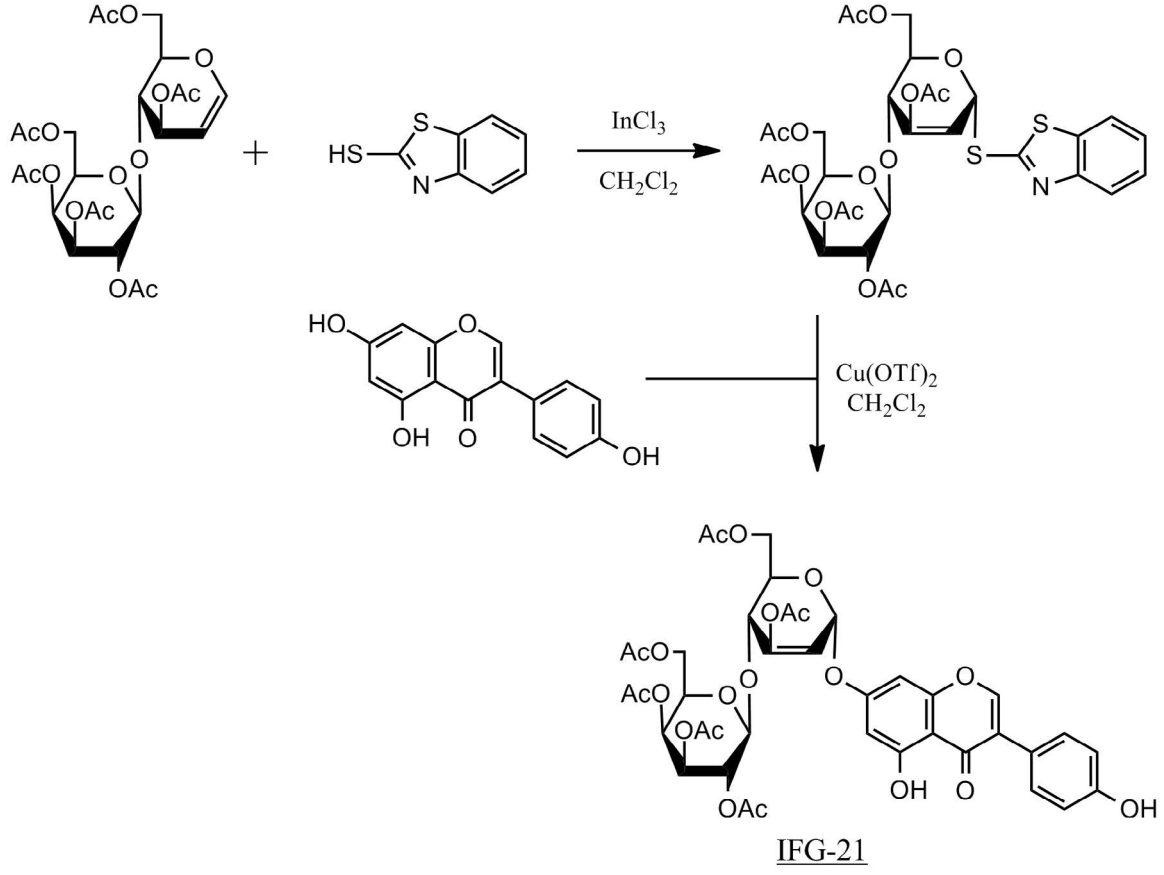


Scheme 15

isobutyl disaccharide carbonate in the presence of Pd(0) afforded a mixture of products containing all four possible diastereomeric glycosides (Scheme 15).

These compounds proved extremely difficult to separate, not only preparatively but even by analytical HPLC. The main component, isolated by meticulous crystallization (IFG–21) unexpectedly exhibited interesting cytotoxic properties against selection of cancer cell lines, while the three remaining isomeric products showed no such activity. Naturally, this finding prompted more effort towards synthesis of hitherto unknown class of isoflavone glycosides. At first, an influence of the anomeric substituent on the reaction stereo– and regio– selectivity was examined and it soon turned out that IFG–21 could be prepared more effectively by using hex–2–enose–2–mercaptobenzothiazole derivative as glycosyl donor (Scheme 16).

Since it seemed that the presence of unsaturated sugar moiety renders isoflavone conjugates some specific biological activity traits, which are absent in the parent natural products, a question arose if these features could be preserved with some structural modifications, which offer better prospects for design of a facile and scalable chemical synthetic process for their preparation. Consequently, a project was formulated for testing syntheses and biological activity of new derivatives, in which hex–2–enose moiety is connected to isoflavone ring system through carbon chain spacer. Additionally, possibilities and effects of replacement of oxygen atom anomeric linkages by more stable carbon connections were also examined. The results can be summarized as follows: at first, simple experimental protocols

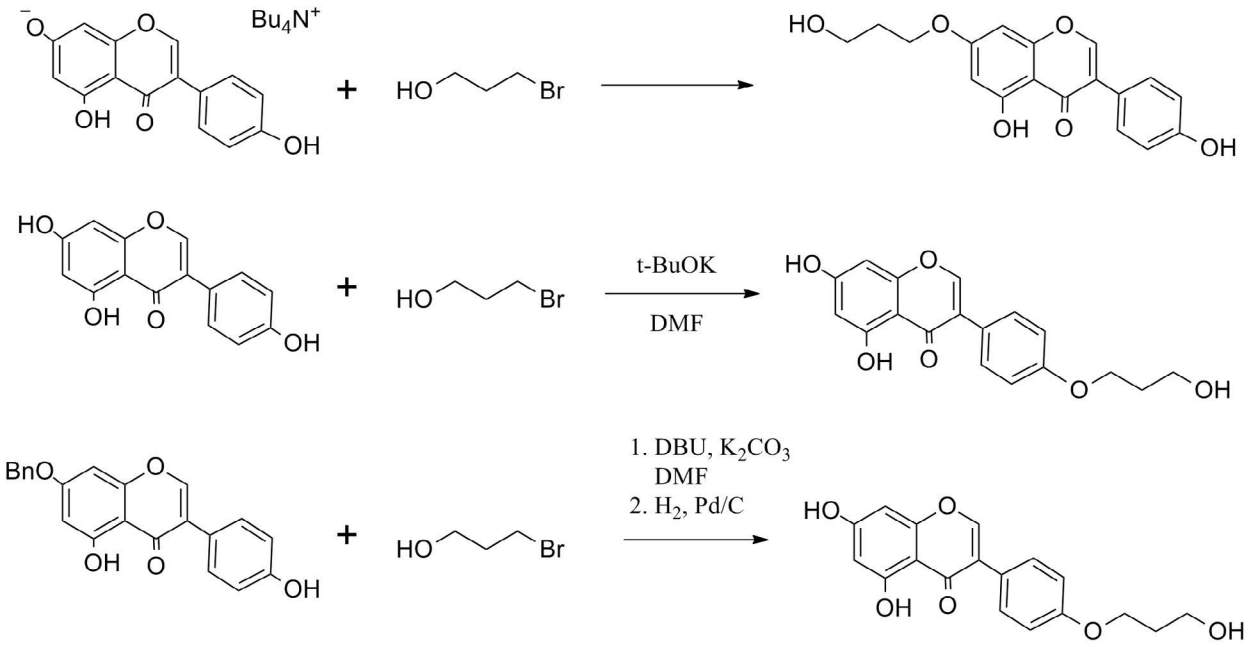


IFG-21

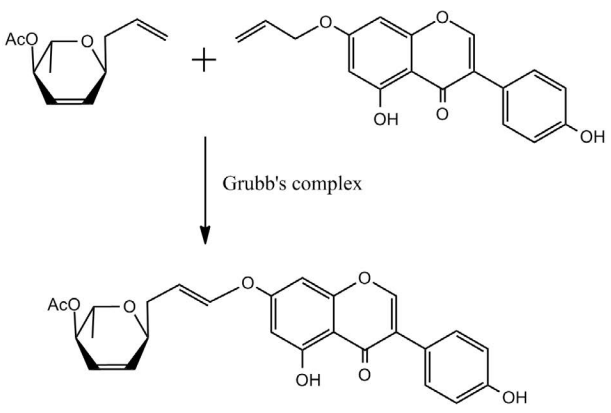
Scheme 16

have been developed, in which availability of selectively protected isoflavone derivatives with ether linkages, which can serve as temporary protecting groups as well as permanent linker for installing structural elements of typical prodrugs or pharmacophore conjugates have been developed [110]. 7–*O*–alkyl derivatives of genistein and daidzein can be obtained by treatment of isoflavone tetra–n–butyl ammonium salt with near stoichiometric amount of an alkylating agent, typically primary bromide containing an ω–protected hydroxyl group for further functionalization. 4–*O*–alkylations can also be performed selectively but require either temporary protection of phenolic 7–OH or double deprotonation of the isoflavone substrate with a strong base, as previously suggested by Wähälä for selective esterifications [109] [111] [112] [113]. Aliphatic alcohols obtained, as illustrated on the Scheme 17 (below), easily undergo FR with variety of glycal derivatives, as well as alternative glycosylation procedures. Resulting compounds,which contain aliphatic primary hydroxyl functionality, easily react with acylated glycals (hex–1–enitols) under Lewis acid catalysis, undergoing Ferrier rearrangement. The reaction is highly regio– and stereoselective, affording predominantly glycosides with α–configuration. Both synthetic steps are in principle very simple, but deserve some comments, when applied to a multifunctional substrate like genistein.

Since *O*–glycosides can be easily hydrolyzed by enzymes present in the human body, some focus has been concentrated on attachment of a sugar moiety via a *C*–glycosidic bond, reasoning that their greater stability will improve the bioavailability without affecting the antiproliferative activity. Although *C*–glycosylated flavonoids occur in nature, no practical synthetic alternative to the biogenetic pathway has been developed thus far. Therefore it has been decided to test a linkage between a carbohydrate moiety and a genistein molecule created by an olefin cross–metathesis (CM) applying reaction between easily accessible appropriate terminal olefins, that is, *C*–allyl glycosides and 7–*O*–allylgenistein (Scheme 18).



Scheme 17

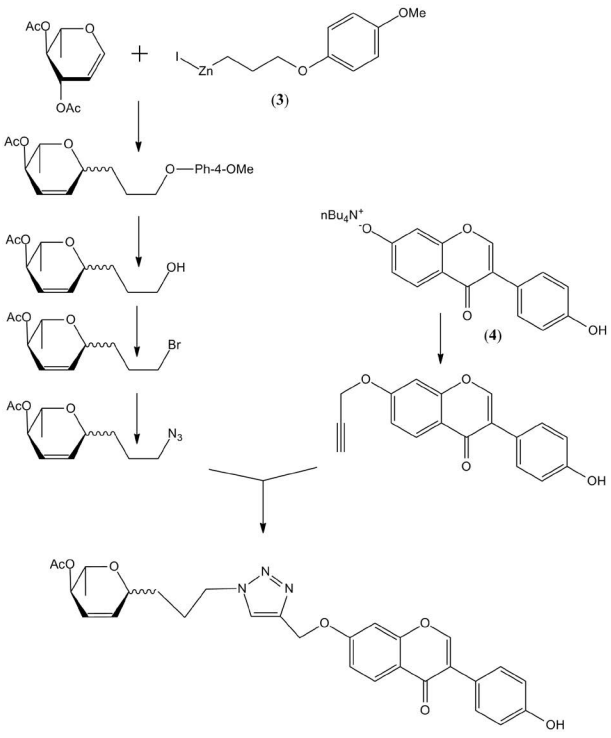


Scheme 18

An alternative idea of linking two principal synthons, namely a sugar moiety and an isoflavone derivative, by C–glycosidic bond, exploiting chemical ligation based on 1,3–dipolar cycloaddition [114] [115] was completed as pictured on Scheme 19 (below).

Scheme 19

As a matter of convenience, it has been decided to place the azido function on an alkyl chain



attached to anomeric position of the pyranose ring and the acetylene group in the form of propargyl phenyl ether at the isoflavone more acidic 7–OH group. To our knowledge, 1–*C*–hydroxypropylation of L–rhamnal has not been described before, despite obvious utility of such synthon. Choice of reagent (**3**) for this particular purpose resulted from trying several alkylzinc iodides, which need to balance low reactivity securing protecting group tolerance, with the ability to perform C–C coupling and effective deprotection of the terminal hydroxyl function [116]. Formation of the azide function in two steps and cyclization to the triazole were carried out according to well known standard procedures [117]. Alkylation of daidzein tetrabutylammonium salt (**4**) with propargyl bromide is expected to be selective, as pK_a values for 7–OH and 4–OH phenolic functions are two orders of magnitude apart, but in fact, most of the alkylating procedures, including phase-transfer catalysis, afford a mixture of regioisomeric products. Our experience indicates that formation of the stoichiometric tetraalkylammoniumsalt prior to the propargylation step is advantageous for regioselectivity and efficiency. The intermediate sugar synthons are poorly separable on TLC but conjugates can be obtained as individual anomers by using silica gel column chromatography.

7. Biological activity of genistein–hexenose conjugates

Secondary metabolites, which have vital environmental and allelopathic functions for a host often occur in their native state as glycosides. Although, the role of a sugar moiety seems to be very important for biological activity of a compound, only few selected categories of natural glycosides (e.g. different classes of antibiotics) have been examined in details for their pharmacological activity, and the studies comparing side by side activities of glycosides and their aglycons are scarce.

Based on a short survey of cases in which

structural modification of natural glycons among low molecular weight secondary metabolites resulted in advantageous pharmacological changes, we postulate that glycosides of natural origin can be quite promising as drug leads. In particular, polyfunctional sugar moieties offer ample opportunities for almost continuous changes in shape, electron density and polarity. Naturally, modifications of pharmacophoric glycosides by replacement of native glycons with glycal derived hex–2–enoses fit this line of reasoning very well.

Despite of considerable advances towards turning enzymatic glycosylations into biotechnological processes, chemical transformations still remain more practical. Particularly, for synthesis of modified glycosides, both in laboratory research and in industry [16].

Among low molecular weight secondary plant metabolites, isoflavonoids (e.g. genistein, daidzein) are recognized as compounds of particular significance for human health as non–nutritional food constituents [109 and references therein]. Recent papers confirm genistein utility in the field of cancer chemoprevention or treatment of osteoporosis and cardiovascular diseases. Genistein comes out of biological processes as a glycosylated species, commonly bearing β–D–glucose (which may be acylated, particularly at C6) at one of the phenolic groups. The glycosidic bond linking glucose to the aglycone is prone to undergo enzymatic hydrolysis mediated by native glycosidases that are widely distributed in mammalian cells and tissues. Importantly, it is possible to introduce diverse sugar groups (e.g. modified or a typical sugars) as well as various types of glycosidic bond to control lipophilicity of a molecule and its susceptibility to enzymatic biodegradation. Consequently, we adopted a plan to compare biological activity within a series of the free aglycone (genistein), its natural glucoside (genistin) and peracetylated genistin, along with some other genistein glycosides, like compounds presented on Schemes 16–19, that bear rather loose resemblance to the natural prototypes. In this chapter we describe biological effects of these compounds, including antioxidant properties and antitumor activity.

Peroxidation of lipids, as well as other membrane components by reactive oxygen species (ROS) leads to the impairment of membrane structure and functions, and in consequence may cause many serious diseases like atherosclerosis, stroke, cardiovascular problems or cancer. Detrimental effects of oxidative damage can be reduced by the presence of different molecules possessing antioxidant, ROS scavenging properties. Flavonoids belong to the best known representatives of this group of substances. When considering the molecular mechanism of flavonoid antioxidant activity exerted on lipid membranes it seems obvious that interaction of flavonoids with lipids must be involved. Since the position of flavonoids within lipid bilayer seemed to be essential for reactivity, several attempts were made to determine the localization of membrane–bound flavonoid molecules and the effects of flavonoids glycosides and glycoconjugates on the membrane permeability. The ability of genistein benzyl and glycosylated derivatives to permeabilize the liposome membrane was studied by calcein–leakage method [118]. Genistein derivatives decreased liposome membrane integrity in calcein release and molecular modeling study. All studied derivatives appeared to be more effective than their parent compound — genistein. When comparing the effect of glycosylated derivatives on membrane permeability, it was recognized that for IFG–21 the increase of permeability appeared at lower concentrations, but the maximal increase (saturation level) of the calcein leakage observed at high flavonoid concentrations was stronger for Ram–3.

During our study on the synthetic glycosides of genistein, we have found that some of 2,3–unsaturated pyranosides (Fig. 2, type A) possess a rather unusual and potentially useful ability to interfere with microtubule dynamics and cell cycle progression. Structural analogs of unsaturated 7–*O*–genistein pyranosides selected as the first group of derivatives for biological activity study were the compounds with an aliphatic carbon spacer, which separated a phenolic part and a carbohydrate moiety (Fig. 2, type B). These compounds have retained antiproliferative activity, which prompted the design of further analogs. Since one of the principal problems of glycoside application in medicinal chemistry relates to the question of their integrity in biological media, it appeared that desired modification should lead to replacement of the *O*–glycosidic by the *C*–glycosidic bond (Fig. 2, type C) [119] [120] [121].

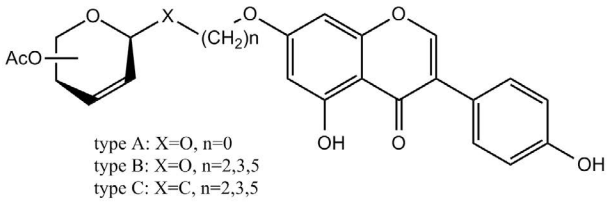


Figure 2

Among type A derivatives obtained so far in our laboratories from intermediate anomeric carbonate esters, according to the general procedures presented before [122] [112], 7–*O*–(2,3,4,6–tetra–*O*–acetyl–β–D–galactopyranosyl)–(1→4)–(6–*O*–acetyl–hex–2–ene–α–D–erythropyranosyl) genistein, shortly named IFG–21, exhibited not only higher antiproliferative potency, but also distinctly different mechanism of action than parent isoflavone. This feature has also manifested itself, although to lesser extent, in the type B conjugates.

The genistein derivatives, glycosides of 2,3–unsaturated sugars, were found to inhibit proliferation of multiple cancer cell lines at a concentration significantly (5–10 times) lower than a parent compound, what makes them attractive objects, both for pharmacological studies and as lead compounds for further modifications. On the basis of structural features and calculated lipophilicity coefficient clogP the derivatives were classified as hydrophilic (i.e. those containing free sugar moiety) or lipophilic (i.e. those with alkylated or acylated sugar hydroxyls). The *in vitro* cytostatic and cytotoxic studies showed hydrophilic glycosides to be practically inactive against human cancer cell lines when compared to the free aglycone. On the contrary, lipophilic glycosides were significantly more active than the parent isoflavone although the correlation between clogP and the activity was not clear. On the basis of GI_{50} and LC_{50} values, two of the most active glycosides were found to be several times more potent in their cytostatic and cytotoxic effect than genistein. Additionally, all lipophilic glycosides exhibited different mode of action in comparison to genistein. It may suggest that these compounds do not undergo rapid biodegradation, either in culture media or inside cells, and exert their biological effects primarily as intact molecules. Of all tested compounds, the most active were found to be IFG21 and IFG30. Apart from some increase in lipophilicity, these glycosides demonstrated the following structural features: acetylated sugar hydroxyls, double C–C bond in the sugar molecule binding directly to aglycone, a configuration of genistein–sugar glycosidic bond, and localization of the sugar substituent at 7–OH position in genistein molecule. These features may be summarized as contributing to relatively weak glycosidic bond, which seems to be the condition for an enhancement of cytostatic/cytotoxic activity to tumor cell lines.
In order to assign the mechanism of antiproliferative activity of IFG–21 we investigated the influence of genistein and

IFG–21 on the cell cycle progression [123]. In subconfluent control cells we have observed two main populations of cells with different DNA content: 60% of cells were in G1phase of a cell cycle (2CDNA), and around 20% of cells were in G2/M phase of a cycle (4CDNA). The treatment of cells with IFG–21 resulted in a significant accumulation of cells in G2/M phase of a cycle (4CDNA) as compared to cells treated with genistein. Treatment of cells for 24 h with IFG–21 led to the increase of the mitotic index from 6%, observed in control, to almost 30%. In contrast to significant increase of the mitotic index values after IFG–21 treatment, the mitotic index in cells treated with genistein was lower than in untreated control, despite the dose of genistein used in the experiment caused remarkable accumulation of cells in G2 phase (4C DNA containing cells). Moreover, in control cells, most of mitotic spindles had two regular poles, and genistein did not influence their structure even at high concentration, while IFG–21 induced aberrant, mainly multipolar mitotic spindles. The fraction of cells containing aberrant mitotic spindle increased in a IFG–21 dose–dependent manner and already at 5 μM most of mitotic figures were aberrant.

The genistein derivative IFG–21 have shown remarkably distinct properties from the native isoflavone in experiments with tumor cell lines, suggesting different mechanism of biological action [122]. Detailed studies of the mechanism of action of IFG–21 provided evidence that the cytotoxic effect of this compound is almost exclusively the result of induction of microtubule depolymerization and subsequent mitotic arrest [123]. This novel mechanism of flavonoid derivative action was further confirmed by Priebe group [124] (unfortunately, unauthorized change of the studied compound symbol took place in the process).

Interestingly, some structural changes in IFG–21, which preserve the hex–2–enose moiety, result in retaining significant features of the tubulin related mechanism of action [111]. Thus, 6–deoxy hexenose Ram–3 (Type B isoflavone conjugates, structures on Fig. 3) could exhibit the same mode of action as IFG–21, although to a lower extent.

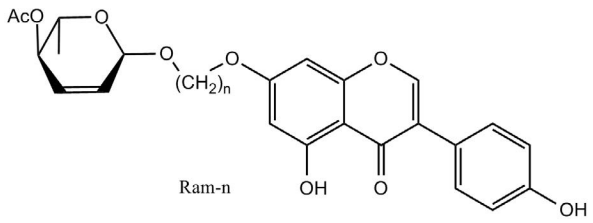


Figure 3

Antiproliferative potential of Ram–3 and genistein was assessed in human cell lines of different origin: glioblastoma, breast, colon, prostate, lung and stomach cancers. In all tested lines we observed stronger inhibition of cell growth by Ram–3 than by genistein. The susceptibility of different cell lines to Ram–3 differed to some extent, but we did not observe any organ specific activity of Ram–3. We also tested the influence of Ram–3 on the cell cycle in four cell lines: HCT116, AGS, A549 and DU145, and we found cell cycle arrest in G2/M phase. Ram–3 inhibited tubulin polymerization in a concentration dependent manner and thus, it can be classified as a new microtubule destabilizing agent. The unique ability of this glycoconjugate to affect microtubules of a spindle indicate, that lipophilic sugar in Ram–3 does not act simply as a carrier, delivering genistein into cells, but it is a structural element essential for antimitotic activity.

However, minor changes introduced to the molecule, such as decreasing or increasing the distance of a sugar moiety and the aglycon, may substantially alter biological properties of a derivative. For example, Ram–2 and Ram–5 derivatives, bearing the same sugar substituent as Ram–3, but obtained through attachment of two C–atoms side chain or five C–atom side

chain to C–7 of genistein, lost the ability to affect microtubule array. Similarly, a series of lactose derivatives (Lac–2, Lac–3, Lac–5), in which the sugar moiety identical as in IFG–21 is linked to genistein via a C2–C5 spacer, did not influence microtubules.

We further explored the effects of regioisomeric substitution of genistein on biological effects of derivatives in cancer cell lines [125]. 2–hexenose derivatives of genistein regioisomerically substituted at 7–*O*– and 4'–*O*– were obtained by methods outlined above. Some of the derivatives substituted at 4'–*O*- inhibited cell proliferation very potently, similarly to their 7–*O*– substituted counterparts. We have demonstrated, however, that antiproliferative effects of 4'–*O*– substituted derivatives of genistein are related to the mechanism different than that described for 7–*O*– substituted regioisomers [125, 111]. Antiproliferative effects of 4'–*O*– derivatives were mediated at least in part by arrest of cell-cycle progression at G1–S. The presence of a substituent at the C4'position of the ring B in genistein correlated to a p53–independent G1 cell–cycle arrest. C4' substituted genistein derivatives did not induce any DNA lesions, there was no ATM kinase activation, no increase of micronucleated cells above the control level and no DNA damage, as revealed by comet assays or histone H2A.X staining, which all occurred in genistein treated cells. The results provided a proof of concept supporting previous observation that hydroxyl group at C4' of genistein is crucial for inhibitory activity of a molecule against topoisomerase II. In contrast, the substitution of genistein with a bulky sugar group at C7, with free hydroxyl present at C4', did not abolish the ability of a molecule to interact with topoisomerase II, as was shown in the example of IFG–21; however, since the molecule affected microtubules with high affinity, biological effects related to topoisomerase II inhibition were not observed in that experimental system [126].

Our results indicate that regioisomeric substitutions at 4'–*O*– and 7–*O*– are key regulators of the mode of isoflavonoid action. Genistein derivatives substituted with a bulky group at C4' block a cell–cycle at G1 phase, despite the presence of a hydroxyl at C5 [125], while those substituted at 7–*O*– (with unmodified hydroxyls present at C5 and C4') cause cell cycle arrest in G2 phase [111, 120] or in mitosis [111, 123] (Fig. 4).

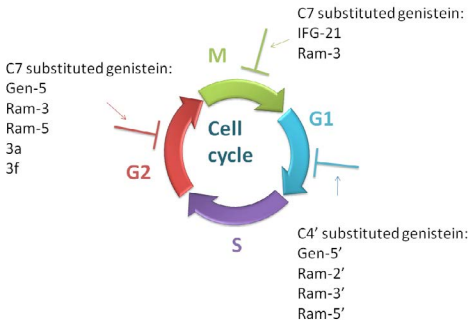


Figure 4

It can be concluded that newly designed synthetic glycosides of genistein, containing a hex–2–enose moiety, derived from glycals via direct application of FR are not just a source of active aglycone (like it is postulated in case of genistin), but in some cases they show significantly higher activity and exhibit individual profiles of biological action. In general, lipophilicity factor was singled out as promoting cytotoxic and antiproliferative activity. Moreover, in specific cases, new molecular mechanism of action, never before observed in flavonoids was uncovered.

8. Conclusions

Natural sugar conjugates of flavonoids in general and isoflavones in particular, have likely evolved in Nature as means of interspecies interaction, with additional function as UV screens and optical signals, all combined in molecules that are better suited for systemic

trafficking than corresponding aglycones, which can be considered as prototype secondary metabolites [91] [92]. Incidentally, some of these compounds have pronounced biological effects on mammalian physiology like for example well studied estrogenic action. Much evidence has been gathered that secondary metabolites from isoflavone class have numerous biological targets, but their interactions are reversible and corresponding affinities to biomacromolecules are rather low. It is reasoned that these biological activities could be tuned up, in terms of efficacy and selectivity, by carefully designed structural changes. A replacement of natural monosaccharides in isoflavone conjugates by hex–2–enoses, attached either directly to 7–*O*–phenolic group or through aliphatic chain spacer, is a step in this direction. Exploration of various types of connections, utilizing selected heteroatoms or switching oxygen for carbon offer plethora of possibilities as far as metabolic susceptibilities of such constructs are concerned. Biological results presented above clearly indicate that products of chemical synthesis, which generally mimics biogenetic glycoconjugation but install hexenose moieties in the glycon part, demonstrate new and promising biological activity features which can be straightforwardly connected to the presence of an unsaturated pyranose.

9. Acknowledgements

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