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# The Flavanolignan Silybin and its Hemisynthetic Derivatives, a Novel Series of Potential Modulators of P-Glycoprotein

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**Abstract**—A new series of potential flavonoidic modulators of P-glycoprotein activity has been prepared. The flavanolignan silybin was first oxidised to dehydrosilybin and then *C*-alkylated with either prenyl or geranyl bromide. The resulting isoprenoid dehydrosilybins were shown to display high in vitro affinities for direct binding to P-glycoprotein, which ranged them among the best flavonoids ever tested. © 2000 Elsevier Science Ltd. All rights reserved.

P-glycoprotein (Pgp) and related transporters are involved in drug resistance of mammalian cancer cells[1,2] and of a number of protozoan parasites such as *Leishmania tropica*.[3] Pgp belongs to the superfamily of ATP binding cassette (ABC) transporters.[4] Thus Pgp extrudes drugs at the expense of ATP hydrolysis, making the cell resistant because of reduced intracellular accumulation of cytotoxic drugs. Pgp is composed of four domains, two hydrophobic transmembrane domains involved in drug efflux and two cytosolic nucleotide-binding domains (NBDs) responsible for ATP hydrolysis. Pgp interacts with a broad array of lipophilic organic compounds in a very complex manner. In fact, some of these compounds are transported out of the cell whereas some others are not. It is believed that, in addition to ATP binding sites, there is a minimum of three binding sites on Pgp, at least two of them being involved in drug transport.[5]

In order to use simpler models for studying Pgp–drug interactions, one approach is to work on soluble recombinant cytosolic domains. Recently, the *C*-terminal cytosolic nucleotide-binding domain (NBD2) from mouse P-glycoprotein was overexpressed in bacteria and purified.[6] In addition to the ATP binding site, this domain contains a hydrophobic steroid binding region.[7] Flavonoid compounds were shown to interact with recombinant NBD2 in a bifunctional manner, by partly overlapping both sites.[8] However, differences in binding affinities were observed between the various classes of flavonoidic compounds. Flavones and flavonols (Scheme 1) displayed better affinities than corresponding isoflavones and flavanones.[6]

On the other hand, the introduction of hydrophobic isoprenoid,[9] or alkoxy[10] substituents on chalcones further improved the affinity. Preliminary unpublished work indicated surprising results for silybin, a derivative of taxifolin (Scheme 2). Silybin is a naturally occurring flavanolignan from Milk thistle (*Silybum marianum*). In fact, despite being a 3-hydroxy flavanone, silybin still exhibited good binding affinity for NBD2, as possibly due to extensive modification of ring B. Therefore it was expected that hemisynthetic transformation of silybin to its corresponding flavonol, followed by isoprenylation of the aromatic ring A would lead to compounds showing among the best affinities towards NBD2.

Thus silybin **2** (purchased from Sigma-Aldrich) was oxidised[11] (Scheme 2) to dehydrosilybin **3** in 90% yield. In order to get in one step the largest diversity of *C*-isoprenoid isomers of **3**, the latter compound was submitted to alkylation in aqueous alkaline medium under catalytic phase transfer conditions. Prenylation of **3** with prenyl bromide[12] yielded a mixture of 6- and 8-prenyldehydrosilybins **4** and **5** in 2 and 1.5% yields, respectively. Similar geranylation[13] using geranyl bromide

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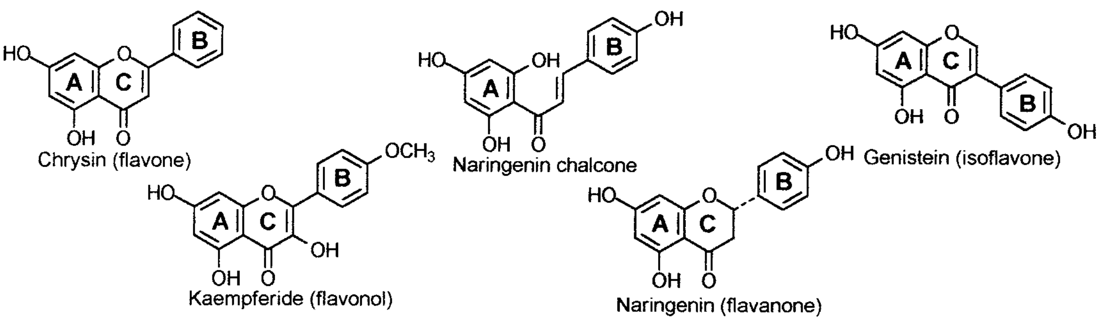
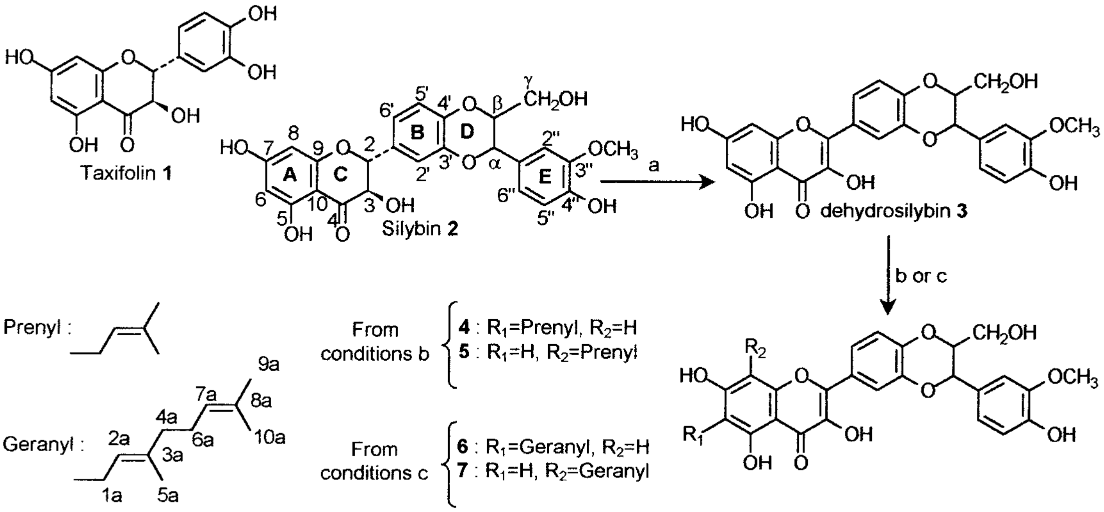


**Scheme 1.** Selected examples of members from the various classes of flavonoids. Classification is based on modifications of ring C.



**Scheme 2.** Structure of taxifolin and hemisynthetic transformations of silybin: (a) KOAc/AcOH/$I_2$; (b) prenyl bromide/tetramethylammonium hydroxide/tetraethylammonium iodide; (c) geranyl bromide/tetraethylammonium hydroxide/tetraethylammonium iodide.

provided 6- and 8-geranyldehydrosilybins **6** and **7** in 1 and 5% yield, respectively. All the compounds were characterised by mass spectrometry, elemental analysis[11–13] as well as NMR spectroscopy (Tables 1 and 2). Signals in Tables 1 and 2 were assigned on the basis of usual combination of 2D NMR experiments (direct $^1$H–$^1$H, direct and long range $^1$H–$^{13}$C correlations).

Compounds **1**–**7** were tested for their binding affinity towards recombinant NBD2 (Table 3). The 3-hydroxyflavanone taxifolin **1**, as expected displayed a low affinity. Its apparent $K_d$ (37.4 μM) was a little bit higher than the value previously obtained with the flavanone naringenin (28.5 μM).[6] However, when the ring B of taxifolin was coupled to a monolignol unit as in silybin **2**, a better affinity for NBD2 was observed. Actually the affinity of the flavanolignan **2** ($K_d = 6.8$ μM) was comparable to that of the flavonol kaempferide ($K_d = 4.5$ μM)[6] previously found as the best flavonoid candidate. Oxidation of silybin **2** to its corresponding flavonol dehydrosilybin **3** increased the affinity by a factor of 3 ($K_d = 2.2$ μM). Prenylation of **3** led to a 6-fold increased affinity in the case of the 6-prenyl derivative **4**, and to a 9-fold increased affinity in the case of the 8-prenyl isomer **5**. A 2-fold further increase in affinity was observed by geranylation, instead of prenylation of **3**, since the geranyl derivatives **6** and **7** exhibited lower $K_d$ values than the corresponding prenyl derivatives **4** and **5**. This effect can be compared to the results recently obtained with series of alkoxy chalcones[10] where the affinity was maximal for substituents with an 8–10 carbons chain length. A higher increase in affinity (7- to 11-fold) was observed in the flavone series[14] when comparing geranyl chrysins to their corresponding prenyl analogues, but the maximal quenching of fluorescence was significantly lower. This might indicate some differences in binding orientation toward ATP and steroid binding sites when comparing flavones and dehydrosilybins.

These isoprenylated derivatives of dehydrosilybin **3**, bind to P-glycoprotein with a considerably higher affinity than compounds such as RU486 ($K_d = 20$ μM),[7] which is known to inhibit Pgp-mediated drug efflux and revert the MDR phenotype of cancer cells.[15,16]

The present results indicate that *C*-isoprenylated derivatives of dehydrosilybin constitute excellent potential modulators of Pgp. More data are required to understand the role of the lignan unit in the interaction, as well as the different effects induced by *C*-isoprenylation in various classes of flavonoids.

**Table 1.** $^1$H NMR data for compounds **3**–**7** ($\delta$ ppm; $J$ Hz), in DMSO-$d_6$ (**3**) or Acetone-$d_6$ (**4**–**7**)

| | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|
| 5-OH | 12.39 *s* | 12.38 *s* | 12.5 *s* | 12.39 *s* | 12.06 *s* |
| 6 | 6.19 *s* | | 6.36 *s* | | 6.37 *s* |
| 8 | 6.45 *s* | 6.66 *s* | | 6.65 *s* | |
| $2'$ | 7.76 br*s* | 7.84 br*s* | 7.90 br*s* | 7.85 *d* (2.1) | 7.88 br*s* |
| $5'$ | 7.11 *d* (9.2) | 7.08 *d* (9.0) | 7.08 *d* (8.7) | 7.09 *d* (9.2) | 7.10 br*d* (9.1) |
| $6'$ | 7.73 *m* | 7.84 br*s* | 7.90 br*s* | 7.85 *dd* (9.1; 2.1) | 7.90 br*s* |
| $\alpha$ | 4.96 *d* (7.8) | 5.06 *d* (8.0) | 5.07 *d* (8.0) | 5.06 *d* (8.0) | 5.06 *d* (8.1) |
| $\beta$ | 4.29 *m* | 4.25 *m* | 4.23 *m* | 4.25 *m* | 4.24 *m* |
| $\gamma_1$ | 3.55 *m* | 3.80 *dd* (12.4; 4.1) | 3.80 *dd* (12.4; 2.2) | 3.80 br*d* (12.4) | 3.81 *dd* (12.4; 2.5) |
| $\gamma_2$ | 3.40 *m* | 3.55 *dd* (12.4; 2.4) | 3.57 *m* | 3.57 *m* | 3.56 *m* |
| $2''$ | 7.03 br*s* | 7.18 *d* (1.9) | 7.18 *d* (1.8) | 7.18 *d* (1.9) | 7.18 *d* (1.8) |
| $5''$ | 6.80 *d* (8.0) | 6.91 *d* (8.1) | 6.91 *d* (8.0) | 6.91 *d* (8.1) | 6.91 *d* (8.0) |
| $6''$ | 6.90 br*d* (8.0) | 7.02 *dd* (8.0; 1.9) | 7.03 *dd* (8.0; 1.8) | 7.02 *dd* (8.2; 1.9) | 7.02 *dd* (8.2; 1.9) |
| 1a | | 3.37 *d* (7.1) | 3.57 br*s* | 3.39 *d* (7.1) | 3.56 *m* |
| 2a | | 5.29 *m* | 5.26 *m* | 5.31 *dt* (7.1; 1.1) | 5.27 *t* (6.6) |
| 4a | | 1.65 *s* | 1.65 *s* | 1.98 br*d* (7.7) | 1.99 *m* |
| 5a | | 1.79 *s* | 1.81 *s* | 1.81 *d* (1.1) | 1.83 *s* |
| 6a | | | | 2.06 *m* | 2.06 *m* |
| 7a | | | | 5.10 *m* | 5.00 *m* |
| 9a | | | | 1.61 *d* (0.9) | 1.54 *s* |
| 10a | | | | 1.56 br*s* | 1.48 *s* |
| $OCH_3$ | 3.78 *s* | 3.90 *s* | 3.90 *s* | 3.90 *s* | 3.90 *s* |

**Table 2.** $^{13}$C NMR data for compounds **3**–**7** ($\delta$ ppm), in DMSO-$d_6$ (**3**) or Acetone-$d_6$ (**4**–**7**)

| | 3 | 4 | 5 | 6 | 7 | | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 144.9 | 145.3 | 145.7 | 145.4 | 145.7 | $1''$ | 127.2 | 128.5 | 128.5 | 128.4 | 128.5 |
| 3 | 136.3 | 136.6 | 137.2 | 136.7 | 135.6 | $2''$ | 111.7 | 111.4 | 111.3 | 111.4 | 111.3 |
| 4 | 175.9 | 176.1 | 171.0 | 176.1 | 176.4 | $3''$ | 147.6 | 148.1 | 148.0 | 148.1 | 148.0 |
| 5 | 160.6 | 158.4 | 159.5 | 158.5 | 159.4 | $4''$ | 147.0 | 147.6 | 147.6 | 147.6 | 147.6 |
| 6 | 98.2 | 111.3 | 98.3 | 111.4 | 98.3 | $5''$ | 115.3 | 115.3 | 115.3 | 115.3 | 115.3 |
| 7 | 164.0 | 162.5 | 161.9 | 162.4 | 161.7 | $6''$ | 120.5 | 121.2 | 121.1 | 121.2 | 121.1 |
| 8 | 93.5 | 93.5 | 106.7 | 93.5 | 106.8 | 1a | | 21.5 | 21.7 | 21.4 | 21.6 |
| 9 | 156.2 | 155.2 | 154.8 | 155.2 | 154.7 | 2a | | 122.7 | 123.1 | 122.6 | 123.1 |
| 10 | 103.0 | 103.5 | 103.7 | 103.6 | 103.8 | 3a | | 131.2 | 131.8 | 134.9 | 135.6 |
| $1'$ | 123.7 | 124.6 | 126.3 | 124.6 | 124.8 | 4a | | 25.4 | 25.3 | 40.0 | 39.8 |
| $2'$ | 116.1 | 116.7 | 116.8 | 116.7 | 116.9 | 5a | | 17.4 | 17.7 | 15.8 | 16.1 |
| $3'$ | 145.7 | 145.9 | 145.7 | 145.9 | 145.6 | 6a | | | | 26.9 | 26.8 |
| $4'$ | 143.3 | 144.3 | 144.3 | 144.4 | 144.4 | 7a | | | | 124.6 | 124.6 |
| $5'$ | 116.7 | 117.3 | 117.2 | 117.3 | 117.2 | 8a | | | | 131.1 | 131.1 |
| $6'$ | 121.2 | 121.7 | 121.7 | 121.7 | 121.8 | 9a | | | | 25.3 | 25.3 |
| $\alpha$ | 75.8 | 76.8 | 76.7 | 76.8 | 76.7 | 10a | | | | 17.2 | 17.2 |
| $\beta$ | 78.4 | 79.5 | 79.6 | 79.5 | 79.6 | $OCH_3$ | 55.7 | 55.9 | 55.8 | 55.9 | 55.8 |
| $\gamma$ | 60.0 | 61.3 | 61.3 | 61.3 | 61.3 | | | | | | |

**Table 3.** Comparison of the binding affinities to recombinant NBD2 for compounds **1**–**7**. Direct binding to the domain was measured as previously described[8]

| Compound | Apparent $K_d$ ($\mu$M) | $\Delta F_{max}$ (%) | Compound | Apparent $K_d$ ($\mu$M) | $\Delta F_{max}$ (%) |
|---|---|---|---|---|---|
| **1** | 37.4 ± 3.3 | 101.1 ± 4.8 | **4** | 0.37 ± 0.02 | 90.3 ± 1.0 |
| **2** | 6.8 ± 0.3 | 91.0 ± 1.6 | **5** | 0.25 ± 0.01 | 93.4 ± 0.6 |
| **3** | 2.2 ± 0.1 | 91.0 ± 1.0 | **6** | 0.18 ± 0.01 | 83.3 ± 0.9 |
| | | | **7** | 0.12 ± 0.01 | 88.1 ± 0.9 |

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11. Oxidation of silybin. To a solution of 5 g silybin **2** in 250 mL glacial AcOH were added 50 g of freshly fused KOAc. The suspension was cooled (ice bath) and a solution of 5 g $I_2$ in 250 mL AcOH was added dropwise under stirring. After complete addition of the iodine solution the medium was refluxed for 4.5 h. The reaction was stopped by addition of 250 mL iced $H_2O$, followed by solid $Na_2SO_3$ (stirring) until fading of the colour. The medium was extracted with EtOAc and the extract concentrated to dryness. The residue was taken in 250 mL of ethanolic HCl (6% v/v) and refluxed for 40 min. Water dilution of the reaction medium, followed by extraction with EtOAc and concentration of the organic layer yielded 4.45 g of pure dehydrosilybin **3**.

Compound **3**. EI–MS (70 ev) *m*/*z* (rel. int.): 480 [M$^+$] (0.7), 462 (0.5), 343 (1), 322 (2), 302 (6), 284 (4), 283 (3), 270 (2), 205 (2), 181 (11), 180 (77), 178 (16), 164 (29), 162 (11), 152 (14), 151 (17), 150 (47), 149 (17), 147 (18), 142 (11), 138 (18), 137 (100), 136 (9), 135 (39), 133 (6), 131 (19), 126 (6), 125 (7), 124 (60), 122 (11), 119 (23), 110 (21), 109 (37), 107 (19), 103 (16), 91 (32), 81 (14), 79 (11), 77 (28), 65 (16), 63 (12), 55 (22), 51 (17). HRMS (EI): calculated for $C_{25}H_{20}O_{10}$: 480.1056. Found: 480.1041. Elemental analysis: calculated for $C_{25}H_{20}O_{10}$, $1.2H_2O$: C, 59.80; H, 4.49. Found: C, 59.67; H, 4.46.

12. Prenylation of dehydrosilybin. 1 g of **3** is dissolved in 140 mL of 10% aq tetramethylammonium hydroxide. After addition of 0.5 g tetraethylammonium iodide, the solution was placed in an ice bath. 300 μL of prenyl bromide were added under argon and the reaction was allowed to proceed for 10 min. The reaction was stopped by addition of 100 mL 1 M aq HCl and extracted with EtOAc. After concentration of the organic layer, the residue was purified by MPLC on $C_{18}$ using a gradient of MeOH in $H_2O$ as solvent. This yielded three fractions A, B and C. Fraction B, containing the prenyl derivatives was further purified by gel filtration on Sephadex® LH-20 using MeOH as solvent. Yielding 21 mg of 6-prenyl dehydrosilybin **4** (2%) and 18 mg of 8-prenyl dehydrosilybin **5** (1.5%).

Compound **4**. EI–MS (70 ev) *m*/*z* (rel. int.): 548 [M$^+$] (16), 531 (25), 530 (54), 528 (17), 513 (29), 487 (24), 476 (16), 475 (39), 412 (13), 411 (49), 409 (23), 395 (24), 393 (13), 371 (18), 370 (66.2), 368 (27), 355 (23), 354 (27), 353 (80), 328 (17), 327 (64), 316 (39), 315 (100), 180 (36), 164 (23), 162 (33), 150 (35), 147 (22), 139 (17), 138 (21), 137 (83), 135 (29), 124 (70), 123 (29), 110 (63), 109 (57), 91 (26), 81 (27), 78 (21), 77 (28), 65 (18), 64 (22), 63 (18), 56 (40), 55 (26), 53 (18), 51 (23). HRMS (EI): calculated for $C_{30}H_{28}O_{10}$: 548.1682. Found: 548.1691.

Compound **5**. EI–MS (70 ev) *m*/*z* (rel. int.): 548 [M$^+$] (2.7), 531 (2.4), 530 (3.9), 395 (3.3), 370 (8.4), 355 (8.7), 332 (9.2), 314 (13), 194 (22.2), 180 (81), 139 (43), 137 (100), 131 (15.9), 124 (51), 119 (25), 110 (12), 109 (15), 91 (36), 79 (11), 77 (21), 65 (17), 55 (22). HRMS (EI): calculated for $C_{30}H_{28}O_{10}$, 548.1682. Found: 548.1681. Elemental analysis: calculated for $C_{30}H_{28}O_{10}$, $2H_2O$: C, 61.60; H, 5.51. Found: C, 61.90; H, 5.45.

13. Geranylation of dehydrosilybin. 1 g of **3** and 0.5 g tetraethylammonium iodide was dissolved in 40 mL of 20% aq tetraethylammonium hydroxide. 500 μL of geranyl bromide were added under argon and the reaction was allowed to proceed for 60 min. The reaction was stopped by addition of 150 mL 1 M aq HCl and extracted with EtOAc. After concentration of the organic layer, the residue was purified by MPLC on $C_{18}$ using a gradient of MeOH in $H_2O$ as solvent. This yielded five fractions A–E. Fraction D was purified by gel filtration on Sephadex® LH-20 using MeOH as solvent, followed by MPLC on diol-bonded silica using 1% *i*-PrOH in $CHCl_3$ as solvent. This yielded 14 mg of 6-geranyl dehydrosilybin **6** (1%). Fraction B was purified by gel filtration on Sephadex® LH-20 using MeOH as solvent, followed by MPLC on diol-bonded silica using 10% *i*-PrOH in $CHCl_3$ as solvent. This yielded 60 mg of 8-geranyl dehydrosilybin **7** (5%).

Compound **6**. Elemental analysis: calculated for $C_{35}H_{36}O_{10}$: C, 70.06; H, 7.17. Found: C, 69.74; H, 7.44.

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