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Isoflavones with unusually modified B-rings and their evaluation as antiproliferative agents

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ABSTRACT

Six novel isoflavone derivatives along with four known isoflavones were isolated from a culture of a highly nickel-resistant strain of *Streptomyces mirabilis* from a former uranium mining area. The structures of 7-hydroxy-3′,5′-dihydroxyisoflavone (**5**), 5,7-dihydroxy-3′,5′-dihydroxyisoflavone (**6**), 2′-hydroxy-3′-methoxygenistein (**7**), as well as hydroisoflavones A–C (**8–10**) were elucidated by MS and NMR analyses. Compounds **8–10** feature yet unprecedented types of non-aromatic, hydroxylated B rings, which result from plant isoflavone biotransformation. All new compounds display weak cytotoxic but potent antiproliferative activities. The anti-oestrogenic properties of **8** against MCF-7 human breast cancer cell line (GI_{50}: 6 μM) is even higher than the reference compound genistein.

Isoflavonoids are prominent plant metabolites that have gained considerable importance due to their diverse broad biological activities, such as antioxidative, insecticidal, piscicidal, antifungal, antimicrobial and oestrogenic.[1,2] In recent years, soy phytoestrogens, mainly the isoflavones daidzein **1** and genistein **2**, have been increasingly used as dietary supplements for their apparent benefits against breast cancer, cardiovascular disease and postmenopausal symptoms.[3] Various previous studies demonstrated that the oestrogenic/anti-oestrogenic activities of genistein **2** are stronger than those of other isoflavones due to its efficient binding to the oestrogen receptor (ER_α and ER_β).[3,4] This finding propelled a broad interest in phytoestrogens for treating menopausal symptoms and lowering incidence of hormone-dependent diseases, including breast cancer.[5] Here, we report the isolation and structure elucidation of novel isoflavones as microbial biotransformation products that feature unusually modified B-rings, and evaluate their anti-oestrogenic and antiproliferative effects.

In a screening program of microorganisms dwelling in specific and rare habitats[6–8] we investigated various *Streptomyces* spp., including a former uranium mining area exploited from 1952 to 1990 in Eastern Thuringia, Germany. *Streptomyces mirabilis* strain P16B-1 as identified by 16S rDNA analyses, with spore chains of the morphotype Spirales in clusters, spore colour from white to greyish and creamy-white substrate mycelium secreting a brownish, melanoid pigment, was isolated from a bank of a creek in a former uranium mining area near Ronneburg, Germany, and was characterized as one of the strains with highest nickel tolerance.[9] Analyses of a small culture extract (100 mL) indicated the production of new isoflavones. Culture filtrate and mycelium of a large-scale fermentation (200 L) were separated and the broth was fractionated through an amberlite XAD-16 resin column with a gradient of $MeOH/H_2O$. LC–MS analyses of extracts indicated the presence of daidzein (**1**) and genistein (**2**), as well as a series of related compounds as judged from their UV spectra and molecular masses. The crude extracts were subjected to open column chromatography using silica gel, size exclusion chromatography with Sephadex. Final purification with preparative RP-HPLC afforded known isoflavones **1–4**[10–12] as well as six novel congeners (30 mg of **5**, 25 mg of **6**, 2.8 mg of **7**, 5.2 mg of **8**, 4 mg of **9** and 1.9 mg of **10**. Their structures were established on the basis of mass spectrometry and 1D/2D NMR spectroscopy (Fig. 1).

The molecular formula of **5** was determined as $C_{15}H_{10}O_5$ by HRESI-MS (*m/z* 271.0601 $[M+H]^+$) and ^{13}C NMR data. The UV and IR spectra are similar to **1**.

The ^1H NMR data revealed an ABX aromatic system of three proton signals at δ 6.83 (1H, d, J = 2.2 Hz), δ 6.92 (1H, dd, J = 2.2, 8.8 Hz) and δ 8.03 (1H, d, J = 8.8 Hz) ppm, respectively, and a singlet of one proton at δ 8.08 ppm attached to a carbon (δ 154.6), which indicates a chromenone moiety. The low field shifted proton signals

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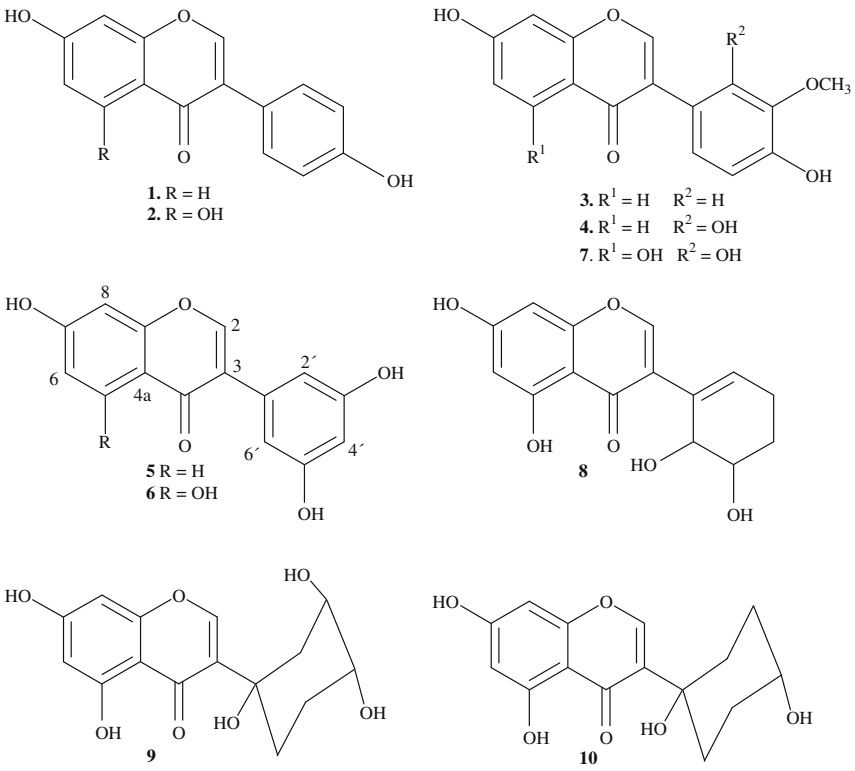

Figure 1. Structures of compounds **1**–**10**.

at δ 8.03 of the ABX system, the presence of a conjugated carbonyl signals at δ 175.1, and the HMBC correlation (H-5 with C-4 and C-8a) suggested that this proton is adjacent to the carbonyl group. This is in accord with the proposed chromenone moiety. Furthermore, the ^{1}H NMR spectrum shows three *meta*-coupled aromatic proton signals, two overlapping signals at δ 6.08, and one at 7.01, all with coupling constants of around 2 Hz; together with the HMBC correlation between H-2′ and C-3, H-6′ and C-2, the ring B of the isoflavone was then assigned. Thus the structure of **5** was elucidated as 7-hydroxy-3′,5′-dihydroxyisoflavone.

Judged from UV and IR spectra, compound **6** seemed to be similar to **2**. HR-ESIMS data (*m/z* 287.0550 $[M+H]^+$) and the ^{13}C NMR spectrum indicated a molecular formula of $C_{15}H_{10}O_6$. Compared to compound **5** the ^{1}H NMR of **6** shows only one alteration: the ABX system is replaced with a meta-coupled system of two aromatic proton signals at δ 6.20 (1H, d, J = 2.1 Hz), and δ 6.30 (1H, d, J = 2.1 Hz). Considering the correlations in the HMBC spectrum and the chemical shift of the two *meta*-coupled proton signals, it was concluded that the ring A of this isoflavone has one more hydroxyl group which is placed in position 5. Accordingly, **6** is a 5-hydroxy derivative of **5**.

The physicochemical properties of compound **7** with a predicted molecular formula of $C_{16}H_{12}O_7$ are similar to those of **6**. The ^{1}H NMR spectrum of **7** shows two *ortho*-coupled aromatic proton signals at δ 6.82 (1H, d, J = 8.4 Hz), and δ 6.44 (1H, d, J = 8.4 Hz), and a new signal corresponding to a methoxy group (3H, δ 3.82) for the ring B. The positions of the substituents (two hydroxy and one methoxy) on the ring B were determined by the correlations of the HMBC spectrum (Fig. 2, and Supplementary data). Compound **7** was thus assigned to be 2′-hydroxy-3′-methoxygenistein.

The molecular formula of compound **8** was determined to be $C_{15}H_{14}O_6$ by HR-ESIMS ($[M-H]^-$ *m/z* 289.0707) and ^{13}C NMR data. The ^{1}H NMR spectrum (300 MHz, CD_3OD) is similar to those of **6** and **7** and shows, in addition to a meta-coupled system of two aromatic proton signals, six non-exchangeable protons and one olefinic proton (δ 5.91). The analysis of ^{13}C, DEPT 135, and HMQC NMR data of **8** revealed, apart from the chromenone moiety, the presence of two methylene C-atoms, three methine C-atoms (one of them sp^2-hybridized and the two others bearing each one an oxygen atom), and one quaternary sp^2-hybridized C-atom. The ^{1}H–^{1}H COSY spectrum showed the coupling system H-2′/H-3′, H-3′/H-4′, H-4′/H-5′ and H-5′/H-6′ for the connectivities of ring B. The junction with the chromenone moiety was defined by HMBC correlation between H-2 and C-3′, H-2′ and C-3. The relative configuration was concluded from NOESY (300 MHz, DMSO-d_6) experiments, which showed the correlations between H-2/ H-2′, OH-5′/ H-6′ and OH-6′/H-5′. Therefore **8** was identified as a modified isoflavone named hydroisoflavone A. This structure is also supported by MS–MS analysis of 291 ($[M+H]^+$) giving daughter ions at *m/z* 273 and 255 ($[M+H]^+$) that indicated the sequential loss of one and two molecules of water.

Compound **9** showed high similarity to **8**. The NMR spectra revealed that the chromenone moiety is maintained and only the ring B is modified. The olefinic proton and the quaternary sp^2-hybridized C-atom are missing, while signals for a quaternary sp^3-hybridized C-atom attached to oxygen and one more methylene C-atom are visible. The molecular formula $C_{15}H_{16}O_7$ as deduced from the HR-ESIMS $[M-H]^-$ (*m/z* 307.0812) and ^{13}C NMR data initially suggested that compound **9** is the hydration product of **8**, but this is not the case. The connectivities of the ring B were determined by the HMBC correlations between H-2 and C-1′, OH-1′ and C-3 for the link with the chromenone moiety, as well as the ^{1}H–^{1}H COSY spectrum, which showed the coupling system H-2′/ H-3′, H-4′/H-5′ and H-5′/H-6′. Finally, this was confirmed by the coupling constant of the proton signals H-2′ (dd, J = 13.83, 3.10 Hz) and H-6′ (td, J = 12.79, 12.74, 3.98 Hz). The relative configuration was established by NOESY experiments, which revealed all expected correlations (Fig. 2). Compound **9** was thus assigned as a novel isoflavone derivative named hydroisoflavone B. Its structure is also supported by MS–MS analysis of *m/z* = 309 ($[M+H]^+$), which

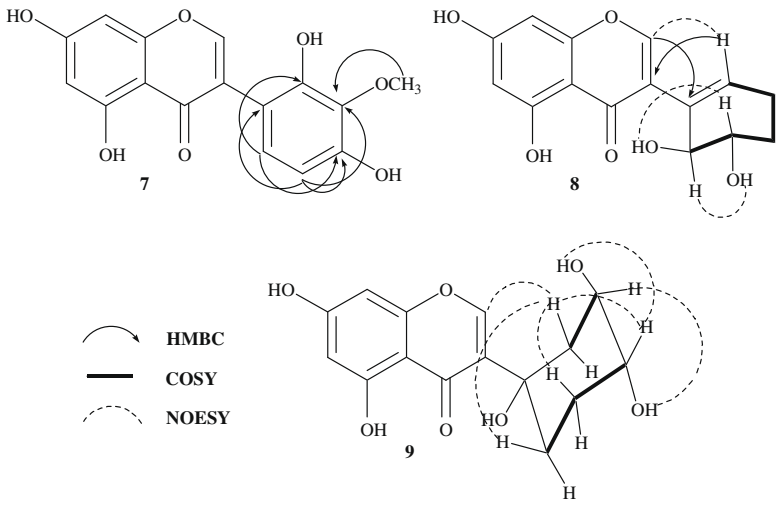

Figure 2. Selected connectivities of compounds **7**, **8** and **9**.

fragments into the daughter ions signals at *m/z* 291, 273 and 255, corresponding to the elimination of one, two and three molecules of water, respectively.

Compound **10** shows also high similarity to **9** except for the replacement of one oxygenated methine C-atom of compound **9** by a methylene C-atom, and the number of carbon atoms signals in the ^{13}C spectrum (15 for compound **9** and 13 for **10**). The HR-ESIMS data (*m/z* 293.1020 $[M+H]^+$) indicated a molecular formula of $C_{15}H_{17}O_6$ for **10**, suggesting a symmetry within its structure. This was confirmed by the presence of eight symmetrical proton signals at δ 1.5–2.4 corresponding to carbon signals at δ 32.9 (2C), and 30.4 (2C). Due to the correlation on the NOESY experiments between H-2 and H-2′, the coupling constant of H-2′ (td, J = 13.23, 12.99, 6.01 Hz), the relative low coupling constant of H-4′ and in accordance with the relative stereochemistry of compound **9**, OH-1′ is placed in equatorial position and OH-4′ in axial position. Altogether, **10** is a deoxy derivative of **9**, named hydroisoflavone C.

Isoflavonoids have been frequently isolated from bacterial and fungal cultures, but their biosynthetic origin has not been unveiled in all cases.[13–15] Only Marchelli and Vining[16] implicated the de novo synthesis of a flavonoid antibiotic (chloroflavonin) by *Aspergillus candidus*, and more recently a filamentous actinomycete (*Streptomyces alni* sp. nov.) producing daidzein (**1**) was characterized.[17] To exclude the rather unlike scenario that *S. mirabilis* is capable of isoflavone biosynthesis, we carried out a series of experiments. First, when changing media, we noted that compounds **1–10** are only detected when soybean meal medium (Medium 1) is used. Second, when isoflavones **1**, **2**, **5** and **6** were added to medium lacking soy bean meal, we could observe the formation of several related isoflavone derivatives. Finally, no incorporation of d5-phenylalanine was observed in a standard isotope feeding experiment, thus ruling out a de novo biosynthesis (data not shown). These findings indicate that *S. mirabilis* transforms plant isoflavones into hydroxylated and reduced derivatives. Out of the new compounds isolated, isoflavones **8**, **9** and **10** are most remarkable as they feature an unusual B ring modification, which is unprecedented in this class of compounds. This is remarkable considering the high number of known isoflavone compounds.

All new compounds were subjected to a broad bioactivity profiling. They displayed only weak antimicrobial and cytotoxic activities, but appeared to be moderate to strong antiproliferative agents (Tables 1 and 2). Compared to the other new isoflavones, compound **8** showed the strongest antiproliferative effect against

Table 1
Antiproliferative and cytotoxic effects of new isoflavones **5–8**

Compound	Antiproliferative effect		Cytotoxicity
	HUVEC GI_{50} (μM)	K-562 GI_{50} (μM)	HeLa CC_{50} (μM)
5	>185	>185	160
6	133	75	134
7	64	48	77
8	17	>172	81
9	>162	>162	>162

Table 2
Antiproliferative effect on MCF-7 and MDA-MB 436 of isoflavones **1–10**

Compound	ER+ MCF-7 GI_{50} (μM)	ER− MDA-MB 436 GI_{50} (μM)
1	122	>196
2	10	17
3	42	75
4	37	68
5	73	169
6	65	>174
7	15	32
8	6	17
9	84	138
10	75	147

the HUVEC cell line with a GI_{50} value of 17 μM. As expected, the isoflavones were more active on the oestrogen receptor positive cell line MCF-7 than to the oestrogen receptor negative cell line MDA-MB 436. Again, compound **8** exhibited the strongest antiproliferative effect on MCF-7 (hormone-dependent) with a GI_{50} value of 6 μM, which is slightly lower than that of genistein (**2**) (Table 2, Fig. 3). This is particularly surprising considering the lack of aromaticity and the clearly different substitution pattern of **8**. In this context it is tempting to speculate that a shape component could be more important than hydrophobicity. The sp^3 centre adjacent to the chromenone in **9** and **10** results in a huge drop off in activity, while sp^2-hybridized olefin in **8** maintains some planarity as in **2**, and both compounds are active at the same order of magnitude. However, the apparently higher log P value[18] of the pseudosugar-like dihydroxycyclohexene substituted **8** (calculated log P value: 0.32) might even be of advantage over **2** (calculated log P value: 1.96) from a pharmacological point of view.

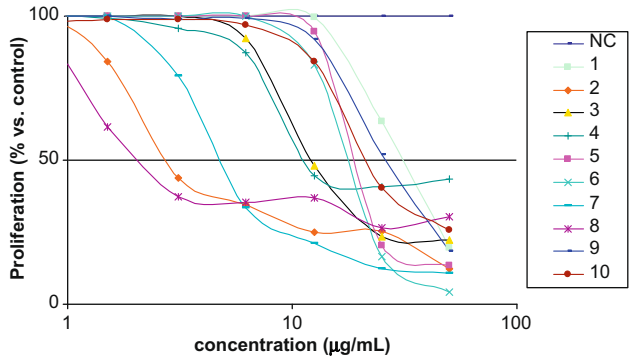

Figure 3. Response curve antiproliferative effect of compounds **1**–**10** on MCF-7 (hormone-dependent).

In summary, we have isolated six new isoflavones from the culture broth of *S. mirabilis*. The structures of all isolated isoflavones, together with the relative configuration of **8**, **9** and **10** were established by MS and NMR studies. Three compounds (**8–10**, hydroisoflavones A–C) possessed an unusual modification of the B ring, which was unprecedented for this class of compounds. All new isoflavones displayed only weak antimicrobial and cytotoxic activities, but exhibit considerably strong antiproliferative effects. Compared to the other isoflavones, compound **8** exhibited the strongest antiproliferative effect, and showed a selective anti-oestrogenic effect on the oestrogen receptor positive MCF-7 human breast cancer cell line compared to its effect on oestrogen receptor negative MDA-MB 436.

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Supplementary data

Supplementary data (general experimental procedure, fermentation and isolation process, ^{1}H and ^{13}C NMR data, biological assays) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.08.084.

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