Structure of Tyrolobibenzyl D and Biological Activity of Tyrolobibenzyls from *Scorzonera humilis*

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A novel tyrolobibenzyl derivative, 1→6-β-D-apiosyl-β-D-glucopyranosyl 4-[2-(4-hydroxyphenyl)ethyl]benzofuran-2-carboxylate **3** (tyrolobibenzyl D) was isolated from *Scorzonera humilis* L. and its structure established by mass spectrometry and 1D- and 2D-NMR spectroscopy. The biological activities of the new compound and related tyrolobibenzyls A–C (**1–2, 4**) and the semi-synthetic peracetyl derivatives of tyrolobibenzyls B (**2a**) and C (**4a**) were assessed. The results revealed no cytotoxic activity against P388 cells and neither anti-bacterial activity against *Bacillus subtilis* nor antifungal activity against *Candida albicans* and *Trichophyton mentagrophytes* for any of the investigated compounds. An evaluation of potential chemopreventive activity of **2, 2a, 4**, and **4a** also revealed no pronounced activity in any of the employed assaying systems.

Introduction

Recently, a new class of naturally occurring bibenzyl derivatives was discovered in the Asteraceae *Scorzonera humilis* L. (Zidorn *et al.*, 2000). Further work on this plant yielded a fourth bibenzyl derivative with the same basic carbon skeleton. The structure of the new compound is reported in this communication. Preliminary investigations using the ^{3}H-thymidine incorporation assay showed no cytotoxic activity against GTB and HL60 cells for tyrolobibenzyls from *S. humilis* up to a concentration of 4 μmol/l (Zidorn *et al.*, 2000). However, investigations on other bibenzyl derivatives, mainly isolated from liverworts, revealed various biological activities, *e.g.* anti-bacterial, anti-fungal, and cytotoxic. Structure activity relationships for anti-fungal bibenzyls indicated that apolar compounds and compounds with a free *p*-phenol group were more active than polar compounds like glycosides and compounds which lack a free *p*-phenol group (Schultz *et al.*, 1990, 1991). (*E*)- and (*Z*)-stilbene derivatives exhibited stronger antifungal activity against *Candida albicans* and *Trichophyton mentagrophytes* than corresponding bibenzyl derivatives (Lorimer *et al.*, 1993). Stilbenes were also found to be more active against brown-rot fungi (*Gloeophyllum trabeum* and *Poria placenta*). In contrast, stilbenes showed no activity against white-rot (*Coriolus versicolor*) but some corresponding bibenzyls were active against this fungus (Schultz *et al.*, 1991).

Prenylated bibenzyls were reported to exhibit anti-bacterial activity (Mitscher *et al.*, 1981; Asakawa *et al.*, 1982). Compounds isolated from the liverwort genus *Radula* showed anti-bacterial activity against *Staphylococcus aureus* at concentrations of 20–30 μg/ml (Asakawa *et al.*, 1982) and similar compounds from the Fabaceae *Amorpha fruticosa* showed anti-microbial activity against *Staphylococcus aureus* and *Mycobacterium smegmatis* at concentrations of 6.25 μg/ml, but no activity against *Escherichia coli*, *Salmonella gallinarum*, *Klebsellia pneumoniae* and *Candida albicans*.

The biological activity of tyrolobibenzyls is of special interest since these are the first natural compounds with a benzofuran-2-carboxylic acid carbon skeleton. Considering that *S. humilis* is

mainly growing in swamps in its Tyrolean distribution area and the comparatively high yield of tyrolobibenzyls in subaerial parts of *S. humilis*, it might be assumed that tyrolobibenzyls serve a specific ecological function for the plants, *e.g.* protection from bacteria or fungi. Therefore we focussed our bio-activity investigations on anti-bacterial and anti-fungal activities. Given the unique structure of this new class of compounds and the fact that *Scorzonera hispanica* L., a related species, is used as a vegetable (black salsify), we also investigated the potential chemopreventive activity of some tyrolobibenzyls. Cytochrome P450 activity in the metabolism of xenobiotica (Murray, 2000), oxidative stress (Kovacic *et al.*, 2001), and inflammation (Fitzpatrick, 2001) are amongst the factors known to contribute to carcinogenesis. Compounds **2**, **2a**, **4**, and **4a** were therefore tested for a) their potential to interact with two enzymes playing a central role in biotransformation of xenobiotica: the phase I enzyme cytochrome P450 1A (Cyp1A) and the phase II enzyme NAD(P)H: quinone oxidoreductase; b) radical scavenging and anti-oxidant properties; and c) their activity in the inhibition of the inflammation enzyme Cox-1 and the inhibition of LPS-mediated iNOS induction in murine macrophages.

Results

Isolation and structure elucidation of Tyrolobibenzyl D

The new natural product **3** was isolated from a methanolic extract of air-dried subaerial parts of *S. humilis* by silica gel column chromatography (CC), Sephadex LH-20 CC and reversed phase (RP18) Lobar CC. On-line mass spectra of **3** displayed signals at *m/z* 575 $[M-H]^-$ and 281 $[M-1'''-6''$-apiosyl-glucose$-H]^-$, congruent with a molecular formula of $C_{28}H_{32}O_{13}$. ^1H and ^{13}C NMR spectra of **3** (Table I) showed signals assignable to a pentose moiety, a hexose moiety and a 4-[2-(4-hydroxyphenyl)ethyl]benzofuran-2-carboxylic acid moiety. ^1H and ^{13}C NMR data of the 4-[2-(4-hydroxyphenyl)ethyl]benzofuran-2-carboxylic acid moiety were very similar to those reported for tyrolobibenzyl A **1** (Fig. 1) (Zidorn *et al.*, 2000). The hexose and pentose moieties were identified on the basis of their NMR data as a β-D-glucopyranose moiety substituted in position 6″ and as a terminal apiose moiety. HMBC crosspeaks from the anomeric proton of the apiose moiety (H-1‴) to C-6″ of the glucose moiety and from the anomeric proton of the glucose moiety to the carbonyl of the tyrolobibenzyl moiety revealed that **3** was the 1‴

Table I. NMR data of tyrolobibenzyl D **3**.[a]

Position	^1H NMR	^{13}C NMR[b]	Position	^1H NMR	^{13}C NMR[b]
2		165.1 s	*glucose moiety*		
3	6.09 1H, s	94.4 d	1″	5.28 1H, δ (7.5)	101.1 d
3a		114.8 s	2″	3.61 1H, m[d]	74.3 d
4		142.7 s	3″	3.51 1H, m[d]	78.2 d
5	6.99 1H, m[d]	129.5 d	4″	3.41 1H, m[d]	71.2 d
6	7.44 1H, dd (8.0, 8.0)	133.1 d	5″	3.74 1H, m[d]	77.5 d
7	7.21 1H, dd (8.0, 1.0)	116.3 d	6″	4.07 1H, br d (10.5)	68.8 t
7a		156.1 s		3.70 1H, m[d]	
COO		168.7 s	*apiose moiety*		
α	3.80 1H, dd (12.5, 5.5)	39.1 t	1‴	5.00 1H, d (2.5)	111.3 d
	3.11 1H, dd (13.0, 7.5)		2‴	3.92 1H, d (2.5)	78.2 d
β	2.91 1H, m	38.4 t	3‴		80.5 s
	2.79 1H, m		4‴	4.02 1H, d (9.5)	75.0 t
1′		133.8 s		3.77 1H, m[d]	
2′/6′	6.99,[c,d] 6.98[c,d] 2H	130.6 d	5‴	3.57 2H, s	65.7 t
3′/5′	6.68,[c] 6.67[c] 2H	116.1 d			
4′		156.3 s			

[a] Measured in methanol-d_4 at 500 and 125 MHz, respectively. Spectra are referenced to solvent residual and solvent peaks of methanol at δ_H = 3.31 ppm and δ_C = 49.0 ppm, respectively.
[b] Multiplicities derived from DEPT experiments.
[c] Most intensive signals of an AA′XX′ spin system.
[d] Overlapping signals.

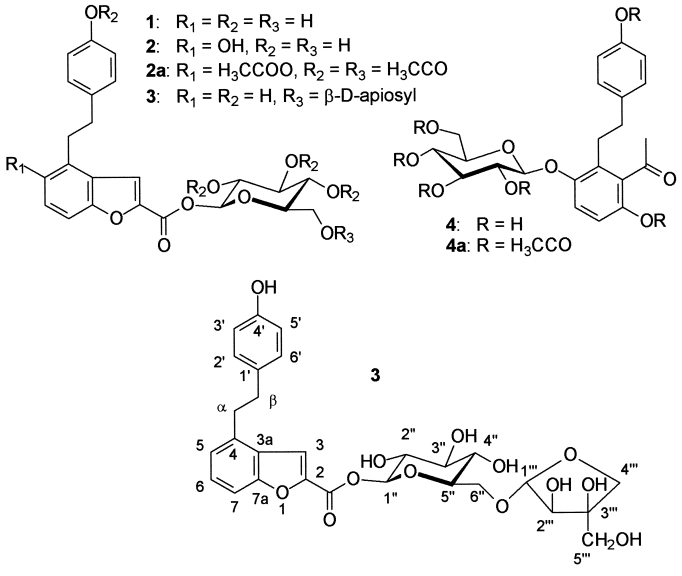

Fig. 1. Structures of tyrolobibenzyls A (**1**), B (**2**), C (**4**), D (**3**), and the peracetyl derivatives of tyrolobibenzyls B (**2a**) and C (**4a**).

→6″-apiosyl derivative of tyrolobibenzyl A (**1**) (Fig. 2).

NMR data of the 1‴→6″-apiofuranose-β-D-glucopyranosyl moiety were in perfect agreement with NMR data reported for 1′→6-β-D-apiofuranosyl-β-D-glucopyranosyl derivatives (Nagai *et al.*, 1986; Gross *et al.*, 1987; Wang *et al.*, 1999; Řezanka *et al.*, 2000). The D-configuration of the terminal apiose moiety was finally proven by the fact that the pair of protons in position 5‴ showed isochronic signals and β-configuration of the anomeric carbon was proven by the ^{13}C NMR shift value measured for C-1‴ (δ_C = 111.3) and by comparing these findings with literature data of the three other possible apiofuranosyl-isomers (Ishii and Yanagisawa, 1998). Thus, the structure of **3** was elucidated as 1‴→6″-β-D-apiofuranosyl-β-D-glucopyranosyl 4-[2-(4-hydroxyphenyl)ethyl]benzofuran-2-carboxylate. In analogy to the three already known tyrolobibenzyl derivatives we name this compound tyrolobibenzyl D.

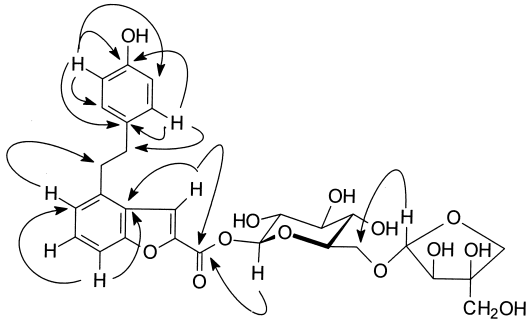

Fig. 2. Important HMBC correlations observed for tyrolbibenzyl D (**3**).

Cytotoxic activity

Cytotoxicity assays using the MTT cell viability assay and P388 murine leukemia cells confirmed the earlier results employing the ^3H-thymidine assay (Zidorn *et al.*, 2000) and showed low or no cytotoxic activity for tyrolobibenzyl derivatives. Compounds **1**, **2**, **2a**, **4**, and **3a** had ID_{50} values of > 25 μg/ml and compound **3** was weakly cytotoxic with an ID_{50} value of 25 μg/ml. Our results revealed furthermore that no other highly cytotoxic compounds are present in considerable amounts in methanolic extracts of subaerial parts of *S. humilis*. Crude methanolic extracts showed no activity up to a concentration of 0.5 mg/ml and the EtOAc phase obtained after partitioning of the crude extract with MeOH/H_2O and EtOAc (Zidorn *et al.*, 2000) had an ID_{50} of 95 μg/ml.

Antimicrobial activity

Agar diffusion assays of **1**, **2**, **2a**, **3**, **4** and **4a** for anti-bacterial activity against *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* showed no growth inhibition at a dosage of 60 μg/

disc. Assays of the crude extract and the EtOAc layer of the crude extract also revealed no antibacterial activity at 1200 μg/disc and 300 μg/disc, respectively.

Likewise, no antifungal activity against *Candida albicans, Cladosporium resinae*, and *Trichophyton mentagrophytes* could be detected at 60 μg/disc for the pure compounds and at 1200 μg/disc and 300 μg/disc for the crude extract and its EtOAc layer, respectively.

Potential chemopreventive activity

Compound **4a** was not testable in any of the assaying systems because of its poor solubility in the test media.

Compounds **2**, **2a** and **4** showed only negligible inductor activity on quinone reductase with IC_{50} values above 50 μmol/l. The influence on the enzyme Cyp1A was weak (13 and 18%, respectively) for compounds **2** and **4** and moderate for compound **2a** (38%) in concentrations of 5 μmol/l.

Compounds **2** (37%) and **4** (15%) showed moderate and weak radical scavenger activity, respectively, in the DPPH assay in concentrations of 250 μmol/l. In contrast, the acetyl derivative **2a** showed no measurable radical scavenging activity in this concentration. All three tested compounds showed no measurable inhibitory (anti-oxidative) activity in the TPA-induced superoxide formation assay in concentrations of 100 μmol/l.

Inhibitory activity of compounds **2**, **2a**, and **4** on the LPS induced expression of iNOS in mice Raw macrophages, was also negligible with IC_{50} values of above 50 μmol/l. Cox-1 inhibitory activity of the tested tyrolobibenzyls was weak in concentrations of 100 μmol/l with inhibition values of 10% (**2**), 15% (**2a**) and 18% (**4**), respectively.

Discussion

There are some possible explanations for the absence of cytotoxic, anti-bacterial, and anti-fungal activity of tyrolobibenzyls against the panel of cell lines, bacteria, and fungi investigated. The natural tyrolobibenzyls **1–4** are polar compounds and will therefore probably not be able to penetrate cell walls and might therefore be hindered to unfold biological activity. However, the peracetyl derivatives **2a** and **4a** are apolar compounds but also showed no biological activity.

Bibenzyls are very rare compounds in Asteraceae with only one report about 2-[2′-phenylethyl]-resorcin-1-carbonic acid derivatives from the South African *Helichrysum umbraculigerum* Less. (Bohlmann and Hoffmann, 1979). It seems therefore reasonable to speculate that tyrolobibenzyls serve in the defense of *S. humilis* against some micro-organisms not investigated in this study, *e.g.* anaerobic bacteria. Alternatively, tyrolobibenzyls might be precursors of phytoalexins, which are synthesized from tyrolobibenzyls only in case of an infection with phyto-pathogenic bacteria or fungi.

Studies on the distribution of the tyrolobibenzyls in the genus *Scorzonera* and in related genera of the subtribe Scorzonerinae are under way.

Materials and Methods

Plant material

S. humilis was collected near Vils/Tyrol/Austria in May 1998. A voucher specimen is preserved in the Herbarium of the Department of Pharmacognosy.

*Isolation of compound **3***

Freeze-dried subaerial parts of *S. humilis* (72.9 g) were exhaustively extracted with MeOH to yield 26.9 g crude extract after evaporating the solvent *in vacuo*. The crude extract was fractionated by silica gel CC using a gradient of CH_2Cl_2 and MeOH. Fractions containing **3** were further separated by repeated Sephadex LH-20 CC using MeOH as eluant. Finally **3** was purified by reversed phase (RP18) Lobar CC using a stepwise gradient of H_2O and MeCN starting with 20% MeCN (v%) and hourly increase of the organic phase by 5% (v%). To separate **3** from co-eluted RP18 material, another Sephadex LH-20 CC using MeOH as eluant was performed yielding 16.3 mg of **3**.

NMR spectroscopy

NMR spectra were recorded on a Varian-Unityplus-500 spectrometer at 500 MHz and 125 MHz, respectively. Spectra were recorded in MeOH-d_4 and referenced to solvent residual signals and solvent signals at δ_H = 3.31 ppm and δ_C = 49.0 ppm, respectively.

ESI mass spectra were recorded in the negative mode on a Finnigan MAT SSQ 7000 mass spectrometer.

Assays of anti-microbial and cytotoxic activity. – Bio-activity assays were performed as described previously (Perry *et al.*, 1999).

Assays of potential chemopreventive activity

Induction of NAD(P)H: quinone oxidoreductase (EC 1.6.99.2) and inhibition of Cyp1A (cytochrome P450 1A, EC 1.14.14.1) activity were assayed as described by Gerhäuser *et al.* (1997) and Crespi *et al.* (1997), monitoring the reduction of MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] to a blue formazan and the dealkylation of 3-cyano-7-ethoxycoumarin to 3-cyano-7-hydroxycoumarin, respectively.

Radical scavenging effects were assessed by a modification of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) microplate system by van Amsterdam *et al.* (1992). Inhibitory (anti-oxidative) activity in the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide radical formation in HL60 cells was tested according to Takeuchi *et al.* (1994).

Inhibition of Cox-1 (prostaglandin G/H synthase, EC 1.14.99.1) activity was measured with a modification of the system described in Jang *et al.* (1997) monitoring the conversion of arachidonic acid to prostaglandins and inhibitory activity on the lipopolysaccharide (LPS) induced expression of inducible nitric oxide synthase (iNOS) in murine RAW 264.7 macrophages was quantified via the Griess reaction as described previously (Ding *et al.*, 1988; Heiss *et al.*, 2001).

*Tyrolobibenzyl D (**3**)*

3 was obtained as a pale yellow amorphous substance, decomposing between 134° and 138 °C, FTIR (micro spectrometry) ν_{max}^{ZnSe}cm^{-1}: 3382, 3362, 3340, 3257, 2925, 2855, 1684, 1601, 1564, 1516, 1450, 1384, 1252, 1209, 1128, 1069, 995, 930, 927; NMR data are given Tab. I.

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