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# Structure Elucidation of the Main Tetrahydroxyxanthones of *Hypericum* Seeds and Investigations into the Testa Structure

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Seeds from *Hypericum* species have recently been identified as an interesting source of xanthone derivatives. Extraction of seeds from *H. perforatum* with MeOH and subsequent concentration via polyamide adsorption yielded a fraction enriched in tetrahydroxyxanthones (THX), which were further semipurified by silica chromatography. Based on tentative structure assignment of the two main THX **X1** and **X2** by NMR a total synthesis was performed for both compounds (THX **1** and **2**, respectively), starting with an *Ullmann* ether synthesis. The synthesized **1** and **2** were characterized via 1D- and 2D-NMR methods as well as by HR-LC-MS analysis and proven to be 1,4,6,7-THX (**1**) and 1,2,6,7-THX (**2**). Final structure assignment of the natural *Hypericum* THX constituents was accomplished by comparing chromatographic and spectroscopic data (LC-MS$^n$ and GC/MS) with those of **1** and **2** which obtained by synthesis. Beyond, investigations into the seeds of *H. perforatum* and *H. tetrapterum* by scanning electron microscopy (SEM) provided insights of the structure of the testa (seed coat), which is established by two cell layers, with the lignified sclerenchyma presumably being the depository of the xanthones.

**Keywords**: *Hypericum perforatum*, *H. tetrapterum*, *seeds*, xanthones, SEM.

## Introduction

Xanthone (9H-Xanthen-9-one) is a base structure belonging to a group of *O*-heterocyclic natural constituents, mainly found in plants [1;2]. Some complex xanthones were also detected as aromatic polyketide antibiotics produced by bacteria (*e.g.* cervinomycin and lysolipin I) [3;4]. Moreover, xanthone structures have recently been identified as lichen or fungal metabolites [5;6]. In the plant kingdom, 24 families have been reported so far to contain xanthones [2], while extensive studies on xanthone chemistry were made *e.g.* in the *Clusiaceae, Gentianaceae, Guttiferae* and *Hypericaceae* families, e.c. [7]. Because of interesting biological and pharmacological features, such as antioxidant, antimicrobial, antiviral, cytotoxic, anticancer, anti-inflammatory, hepatoprotective and MAO inhibitor activities *e.c.*, this compound class is attracting growing attention [2;8-11]. Consequently, the xanthone scaffold is also interesting as a lead structure for the design and development of novel drugs with promising biological activities [12].

In pharmacognosy, the genus *Hypericum* as a source of xanthones with different structures and various biological activities is of particular interest. Among the representatives of this genus, mainly the prominent medicinal plant St. John's wort (*Hypericum perforatum* L.) is currently used in phytomedicine, *e.g.* for treatment of moderate depressive episodes [13; 14]. Pharmacological studies have identified the xanthones and flavonoids, besides naphthodianthrones (*e.g.* hypericin) and phloroglucinols (*e.g.* hyperforin), as active ingredients responsible for the anti-depressant effect of *H. perforatum* [15- 17].

A variety of xanthone derivatives have been analyzed in a number of *Hypericum* species [18; 19]. These may be classified into six structural subgroups: simple oxygenated xanthones (exhibiting OH and OMe fuctional groups), xanthone *O*- and *C*-glycosides, prenylated

xanthones, xanthonolignoids, xanthone dimers and others [9; 20; 21]. Biosynthetically, xanthones are derived from mixed shikimate and acetate pathways: phenylalanine formed from shikimate is oxidized to form benzoic or 3-hydroxybenzoic acid as substrates. Involving enzymes like 3-hydroxybenzoate-CoA ligase, malonyl-CoA and xanthone synthases/hydroxylases [22], the substrates are stepwise converted, with benzophenones being formed as intermediates, into xanthones. Interestingly, recent investigations have revealed xanthones to be produced by plants to protect cells from oxidative damage and as phytoalexins to impair pathogen growth (*e.g.* of microbes) [23; 24]. Thus, this compound class is also of particular interest from a chemical ecology viewpoint.

We recently reported comprehensive phytochemical studies on seeds of two *Hypericum* species (namely *H. perforatum* L. and *H. tetrapterum* FR.) [25]. Besides fatty oil ($CH_2Cl_2$ extract), a MeOH fraction was recovered from *Hypericum* seeds, being characterized by high content of numerous xanthones, with different functional groups and substitution patterns. By means of HPLC-DAD/$MS^n$ and GC/MS xanthone structures with hydroxy, methoxy and sulfonate groups, as well as glycosidic substitution were detected [25]. Among these, two tetrahydroxyxanthones (THX, **X1** and **X2**) were analyzed as main constituents of the seeds of both *Hypericum* species (*H. perforatum* and *H. tetrapterum*). However, based on these data complete structural elucidation of the THX could not be accomplished. Consequently, exact constitution analysis of THX **X1** and **X2** was focused on in the current investigation. In the course of this study a total synthesis of both compounds should be implemented to gather reference material for chromatographic and spectroscopic investigations, as well as for future bioactivity studies. Since structural and morphological investigations into *Hypericum* seeds are scant, we also scrutinized the seeds by scanning electron microscopy (SEM). These investigations provide valuable information concerning the morphology and anatomy of the seeds, thus allowing conclusions with regard to the localization and function of the xanthones.

## Results and Discussion

Complete structure assignment of the main THX in *H. perforatum* and *H. tetrapterum* from a chromatographically purified *H. perforatum* seed extract was performed by NMR, LC-DAD-$MS^n$ and GC/MS experiments. Furthermore, the two THX **1** and **2** were synthesized for the first time and compared with the genuine *Hypericum* THX **X1** and **X2**, regarding their chromatographic and spectrometric/spectroscopic features. In addition, the contents of **X1** and **X2** in the seeds of *H. perforatum* and *H. tetrapterum* were determined via a validated HPLC-DAD method applying external calibration with a synthesized reference standard (1,3,6,7-THX, **3**).

*Isolation, Synthesis and Structure Assignment of the THX from* Hypericum *Seeds*

For the isolation and characterization of THX, dry seeds of *H. perforatum* were first defatted by repeated extraction with $CH_2Cl_2$, yielding a fatty oil fraction (31.7 % of the seed weight). Subsequently, the defatted seeds were extracted with MeOH. The obtained crude MeOH extract (3.63 % of the seed weight) was subsequently purified via polyamide adsorption to yield a fraction (0.88 %) with a high content of THX. The latter was further purified on silica. By elution with a $CH_2Cl_2$/MeOH gradient a THX-enriched main fraction was obtained. By GC/MS analysis of this fraction (see below), a mixture of two main THX **X1**, **X2** with a peak area ratio of 4.2 : 1.0, respectively, was analyzed (data not shown). A sample of this fraction was also investigated by NMR. In the $^1$H-NMR spectrum (see *Fig. 1*) 2 x 4 proton signal groups (2 x 2 singlets (*s*) at $\delta$ 7.41/6.92 and 7.37/6.86 ppm and 2 x 2 doublets (*d*) at $\delta$ 7.18/6.57 and 7.23/8.86 ppm) were identified for both THX. Based on 2D-NMR experiments (*g*HSQC and *g*HMBC, data not shown) and the resulting proton/carbon signal correlations tentative structure assignments suggested 1,4,6,7- and 1,2,6,7-OH-substitution patterns for **X1** and **X2**, respectively.

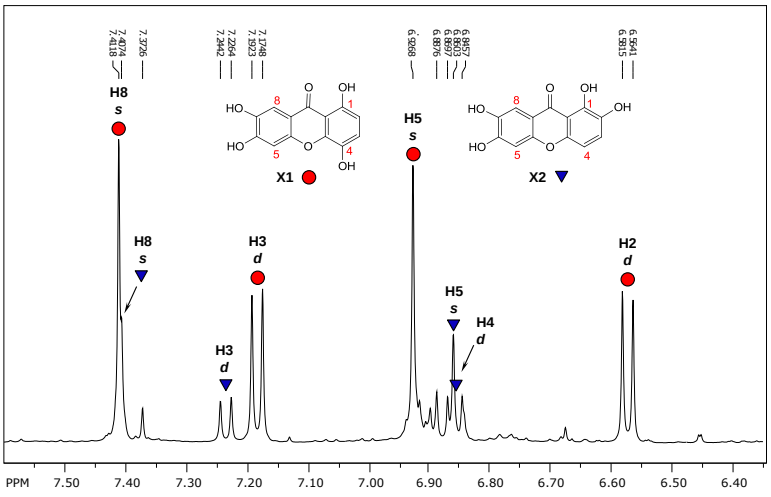
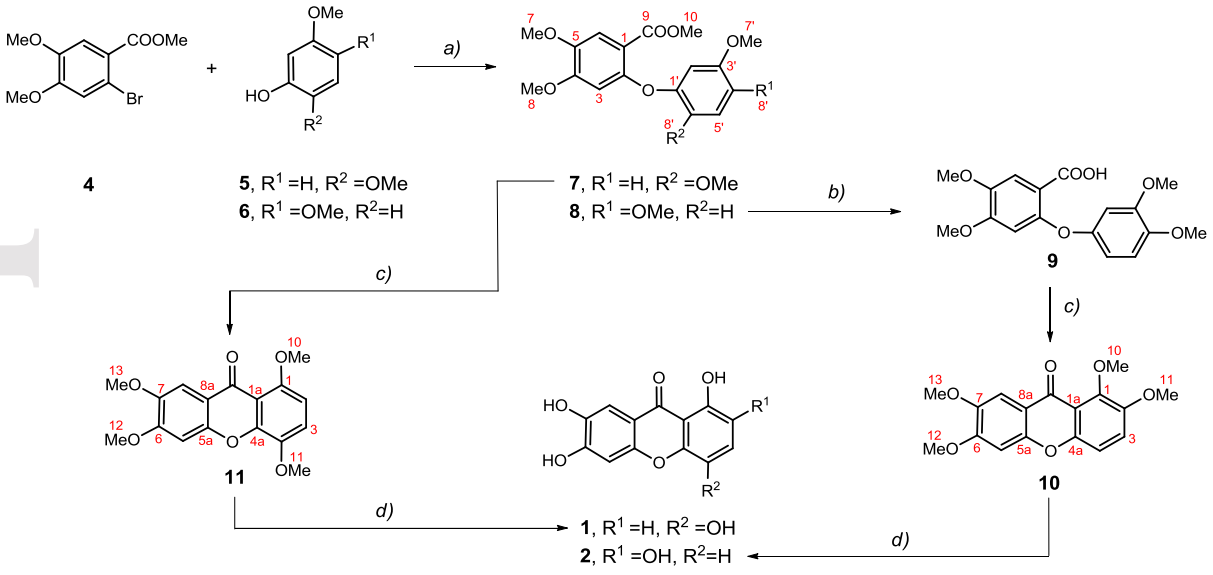



**Figure 1.** $^{1}$H-NMR spectrum of the THX-enriched fraction obtained by purifying a MeOH extract of *H. perforatum* on polyamide and silica, showing proton signals of the two main THX **X1** and **X2** .

To prove the structure assignments, based on the 1D- and 2D-NMR results, an attempt was made to synthesize THX **1** and **2**. By applying an established concept, formerly published for the synthesis of other xanthone derivatives [26; 27] both target structures were synthesized. Consequently, starting with the synthesis of **2**, 2-bromo-4,5-dimethoxybenzoic acid methylester **4** together with the corresponding substituted phenol **6** were converted according to an *Ullmann* reaction into a bi-aryl ether methylester **8** (*Scheme 1*). Hydrolysis of the latter yielded a carboxylic acid intermediate **9**, which was cyclized to the pursuant tetramethoxyxanthone **10** by reaction with LDA in THF. In the final reaction step the protective groups (OMe) were de-methylated by treatment of **10** with $BBr_3/CH_2Cl_2$ to yield 1,2,6,7-THX (**2**). In a similar manner 1,4,6,7-THX (**1**) was prepared, though with the difference that the corresponding *Ullman* condensation product **7** (methylester) was directly transformed into the corresponding xanthone by cyclization, to give **11** (*Scheme 1*). However, this pathway gave lower xanthone yields. This is a reason why the first method is considered preferable.




**Scheme 1.** Synthesis pathway yielding tetrahydroxyxanthones (THX **1** and **2**). *a) Ullmann* reaction (Cu, $Na_2CO_3$, pyridine; reflux ($N_2$), 26 h). *b)* 1. MeOH, THF, 5 M NaOH, $H_2O$, r.t., 96 h; 2. 10 M HCl. *c)* LDA, THF ($N_2$), 0 °C – r.t., 2h. *d)* $BBr_3$, $CH_2Cl_2$, -18 °C – r.t. The red colored arbitrary atom numbering used for NMR assignment.

The structures of the synthesized THX **1** and **2** were confirmed based on 1D- and 2D-NMR-experiments ($^{1}$H-, $^{13}$C-NMR, *g*HSQC and *g*HMBC). The molecular formulae for both compounds were each established as $C_{13}H_7O_6$ and $C_{13}H_9O_6$ by measurement of high-resolution(HR)-LC-MS spectra in the negative and positive ionization modes, respectively. The NMR data of **1** and **2** are depicted in *Tab. 1*.

Notably, the $^{1}$H-NMR shifts of the aromatic protons of synthetic **1** and **2** are in good agreement with the proton signals of the *Hypericum*-THX mixture (THX-enriched fraction obtained from silica chromatography) as shown in *Fig. 1*. However, for further structure assignments of *Hypericum* seed THX and comparison with synthetic **1** and **2**, LC-MS$^{n}$ and GC/MS investigations were performed.

**Table 1.** $^{1}$H and $^{13}$C NMR data of 1,4,6,7-THX (**1**) and 1,2,6,7-THX (**2**) in DMSO-d6 ($\delta$in ppm).

| **Compound** | **1** | | | **2** | | |
|---|---|---|---|---|---|---|
| **Position** | $\delta_C$[a] | $\delta_H$ (*J* in Hz)[b] | HMBC[c] | $\delta_C$[a] | $\delta_H$ (*J* in Hz)[b] | HMBC[c] |
| **1** | 152.39 (C) | - | - | 147.26 (C) | - | - |
| **1a** | 108.45 (C) | - | - | 108.29 (C) | - | - |
| **2** | 108.56 (CH) | 6.57, *d*, (8.7) | C(1), C(1a), C(4), C(4a)*, C(9)* | 148.65 (C) | - | - |
| **3** | 122.50 (CH) | 7.17, *d*, (8.7) | C(1), C(1a)*, C(4), C(4a) | 123.50 (CH) | 7.22, *d*, (8.9) | C(1), C(1a)*, C(2), C(4a) |
| **4** | 137.32 (C) | - | - | 106.09 (CH) | 6.87, *d*, (8.8) | C(1a), C(2), C(4a) |
| **4a** | 144.19 (C) | - | - | 139.60 (C) | - | - |
| **5** | 102.99 (CH) | 6.94, *s* | C(5a), C(6), C(7), C(8)*, C(8a), C(9)* | 102.64 (CH) | 6.86, *s* | C(5a), C(6), C(7), C(8a), C(9)* |
| **5a** | 151.78 (C) | - | - | 152.05 (C) | - | - |
| **6** | 155.17 (C) | - | - | 155.03 (C) | - | - |
| **7** | 144.32 (C) | - | - | 143.93 (C) | - | - |
| **8** | 108.09 (CH) | 7.40, *s* | C(5)*, C(5a), C(6), C(7), C(8a), C(9) | 107.92 (CH) | 7.40, *s* | C(5)*, C(5a), C(6), C(7), C(8a), C(9) |
| **8a** | 112.41 (C) | - | - | 111.77 (C) | - | - |
| **9** | 180.62 (C=O) | - | - | 180.88 (C=O) | - | - |

[a] Recorded at 150 MHz. [b] Recorded at 600 MHz. [c] The asterisks are indicate $^{4}J$ long-range(C-H)couplings.

*LC-MS$^{n}$ Analyses of the THX, Determination of the THX Contents in* H. perforatum *and* H. tetrapterum *Seeds by HPLC*

HPLC and LC-MS have been proven to be best techniques for separation, identification and quantification of naturally occurring xanthones [28; 29]. For qualitative and quantitative analysis of THX **X1** and **X2** a reversed-phase HPLC method, peviously published by *Heinrich* et al. [25], was applied. This method is suitable for the separation of a wide range of xanthone derivatives from *Hypericum* seeds and covers a broad spectrum of different polarities. The method was applied for HPLC(DAD)-MS$^{n}$(ESI$^{-}$) to generate MS$^{n}$ and UV data for comparing the natural THX (**X1** and **X2**) and synthesized **1** and **2**, and to substantiate structure elucidation. *Fig. 2a* shows an HPLC-DAD section of a polyamide MeOH extract of *H. perforatum* seeds with the two main THX **X1** and **X2**, recorded at 260 nm. As expected, the synthesized reference compounds **1** and **2** were congruent with the natural **X1** and **X2** in their chromatographic, UV and mass spectrometric behaviour (*Fig. 2b-e*). Both THX revealed complex UV spectra with a characteristic absorption maximum at $\lambda$ = 392 nm (see inserts, *Fig. 2b,c*). The ESI mass spectra recorded in the negative ionization mode, exhibit the expected molecular ion [M-H]$^{-}$ at *m/z* 259 for both THX **X1** and **X2**. Characteristic losses of 30, 28 and 44 Da (loss of HCOH, CO and $CO_2$, respectively) in collision-induced dissociation experiments resulted in fragment ions at *m/z* 229, 201 and 187 (*Fig. 2 d,e*). These LC-MS data were similar to our previously published data [25]. Finally, *H. perforatum* and *H. tetrapterum* seeds were consecutively extracted with $CH_2Cl_2$ and MeOH according a literature protocol, and the obtained crude MeOH extracts were analyzed via a validated HPLC method [25] to quantitate **X1** and **X2**. Noteworthy, a fivefold higher content of THX **X1** and **X2** was ascertained for *H. perforatum* in comparison to *H. tetrapterum* seeds (see *Tab. 2*). While total THX concentrations (**X1** + **X2**) of 1180 mg/kg in *H. perforatum* and 233 mg/kg in *H. tetrapterum* seeds were

determined, similar THX ratios for **X1** : **X2** of 1: 1.46 (w / w) and 1 : 1.49 (w/w) were found for *H. perforatum* and *H. tetrapterum* seeds, respectively.

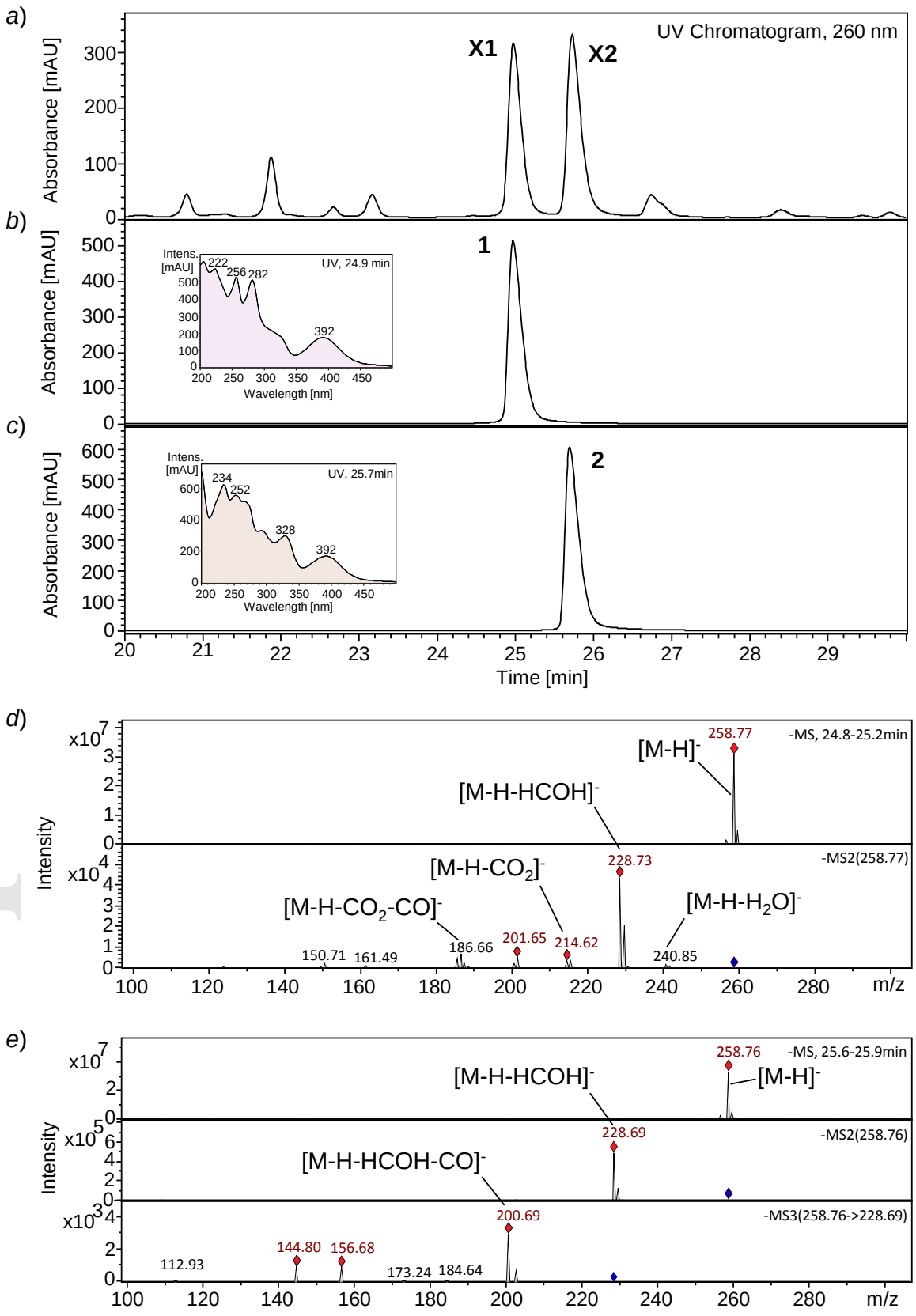


**Figure 2.** Chromatograms and mass spectra of HPLC-DAD-$MS^n$(ESI$^-$) sections of a polyamide MeOH extract of *H. perforatum* seeds and reference compounds. *a)* UV chromatogram of a *H. perforatum* seed extract with the THX **X1**, **X2** and *b)* reference compound: 1,4,6,7-THX (**1**) and *c)* 1,2,6,7-THX (**2**) recorded at 260 nm; the inserts illustrate the UV spectra of the corresponding THX peak. *d)* $MS^n$ data of 1,4,6,7-THX (**X1**; **1**; $C_{13}H_8O_6$, $M_r$ = 260.20 Da). *e)* $MS^n$ data of 1,2,6,7-THX (**X2**; **2**; $C_{13}H_8O_6$, $M_r$ = 260.20 Da).

**Table 2.** Contents of individual THX (**X1**, **X2**) in dry seeds of *H. perforatum* and *H. tetrapterum* ($n$ = 3, analyzed by HPLC/DAD[a]).

| THX | *H. perforatum* Content [mg/kg], (means; s.d.)[b] | *H. tetrapterum* |
|---|---|---|
| **X1** | 478.41 (± 36.13) | 93.49 (± 13.08) |
| **X2** | 700.47 (± 19.02) | 139.72 (± 20.90) |

[a] Detection wavelength $\lambda$ = 260 nm. [b] Determined in crude MeOH extracts by external calibration with 1,3,6,7-THX (**3**) as reference compound according to a literature protocol [25] and calculated based on dry seed weight.

*GC/MS Investigation of the THX in* Hypericum *Seed Extracts*

To provide another analytical method for analysis of the THX and to gain more MS spectrometric information seeds extracts of *H. perforatum* were derivatized with two different reagents and investigated by GC/MS. Thus, for GC/MS analyses the compounds of the polyamide MeOH extract were first methylated by treatment with a $CH_2N_2/Et_2O$/MeOH solution. In the GC/MS chromatogram of this extract 3 peaks were detected after methylation (see *Fig. 3a*). Based on the comparison with formerly synthesized reference compounds **1o** and **11** (*Scheme 1*) the peaks at $R_t$ 50.0 and 51.6 min could be assigned to the tetra-methylated *Hypericum*-THX **X2-Me** and **X1-Me**, respectively (*Fig. 3 a-d*). However, the peak **X0-Me** at $R_t$ 49.0 (*Fig. 3a*) could not be readily assigned. The molecular ion ($M^+$) of the latter revealed an *m/z* ratio at 302, which illustrates **X0-Me** to be tri-methylated THX of undetermined structure. For verification of GC signals allowing comparison of the per-methylated THX under varying laboratory conditions, *Kovats* retention indices [30] were calculated (*Tab. 3*). Using homologous *n*-alkanes ($C_{27}$-$C_{30}$), eluting prior to or after the target compounds (*Fig. 3b*), retention indices were determined (*Tab. 3*). Moreover, the compounds of the polyamide MeOH extract were silylated by treatment with BSTFA and investigated in the same way via GC/MS. Hence, two main peaks at $R_t$ 50.0 and 51.3 min were analyzed in the chromatogram (*Fig. 4a*) of the silylated fraction. By means of silylated reference compounds **1** and **2**, the resulting THX tetra-silylethers **X1-TMS** and **X2-TMS** could be readily assigned (*Fig. 4b, c*). Unexpectedly, a reverse retention of the silylethers of **1** and **2** (*Fig. 4*) compared to their methylethers (*Fig. 3*) was observed, which can also be deduced from the *Kovats* retention indices of the detected compounds (*Tab. 3*). Besides, a minor THX **X3** was identified based on its silylether (**X3-TMS**) at $R_t$ 51.5 min by comparison with reference material and assigned to 1,3,6,7-THX (**3**), (*Fig. 4a, d*). This compound **X3**, also referred to as norathyriol (**3**), has already been reported for *H. perforatum* in the literature [31] and was also detected in *Hypericum* seeds in our former investigations [25]. 1,3,6,7-THX (**3**) and another one, 1,3,5,6-THX, are tetrahydroxyxanthones which have been solely detected in *Hypericum* species so far [19; 31]. Compound **X3** was not further addressed in this study.

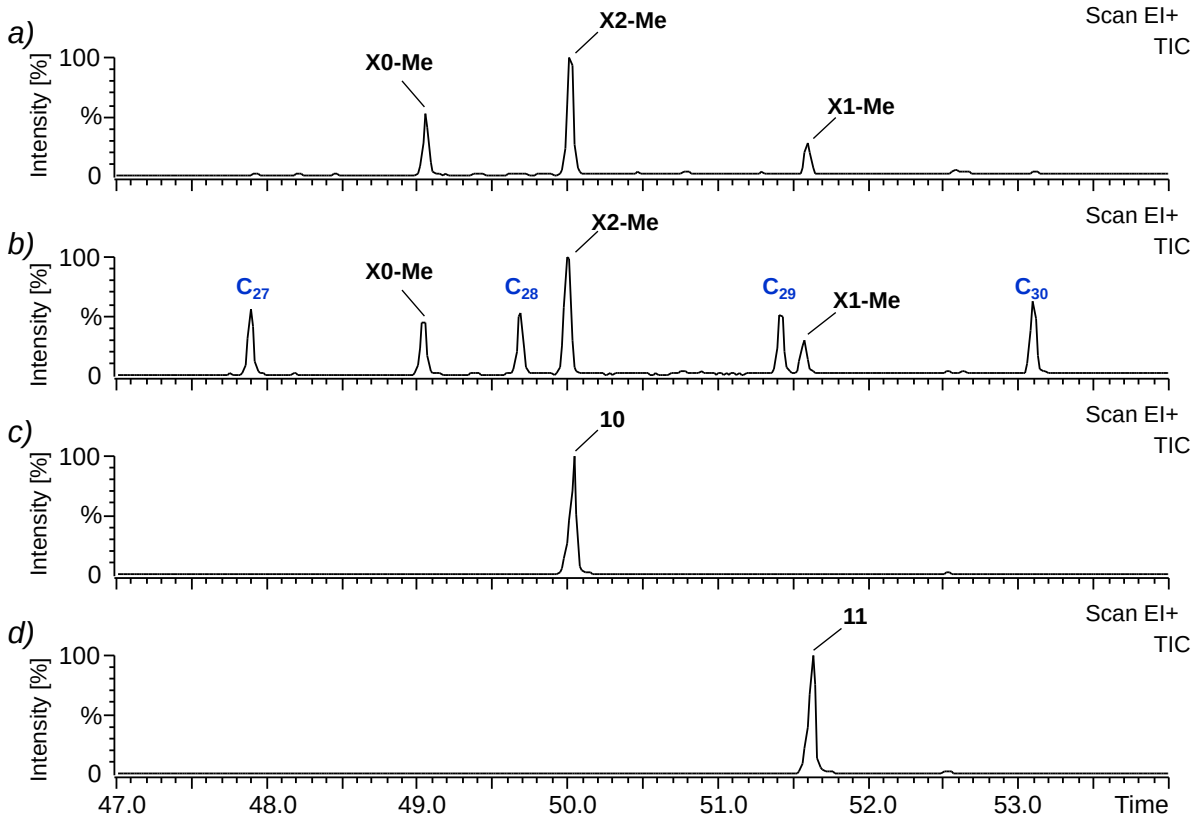



**Figure 3.** Sections of GC/MS profiles (EI) showing the methylated THX of *H. perforatum* seeds. *a)* THX fraction (polyamide MeOH extract), after treatment with $CH_2N_2$. *b)* Identical fraction after addition of an *n*-alkane mixture (**$C_{27}$-$C_{30}$**) for the determination of *Kovats* retention indices (KRI); **X0-Me** designates a peak of a tri-methylated THX of undetermined structure. *c)* Reference compound: tetra-methylated 1,2,6,7-THX (**10**, *Scheme 1*). *d)* tetra-methylated 1,4,6,7-THX (**11**, *Scheme 1*); the tetra-methylated 1,3,6,7-THX (**X3-Me**), assigned with reference compound **12**, co-eluted with **X1-Me** (not shown).

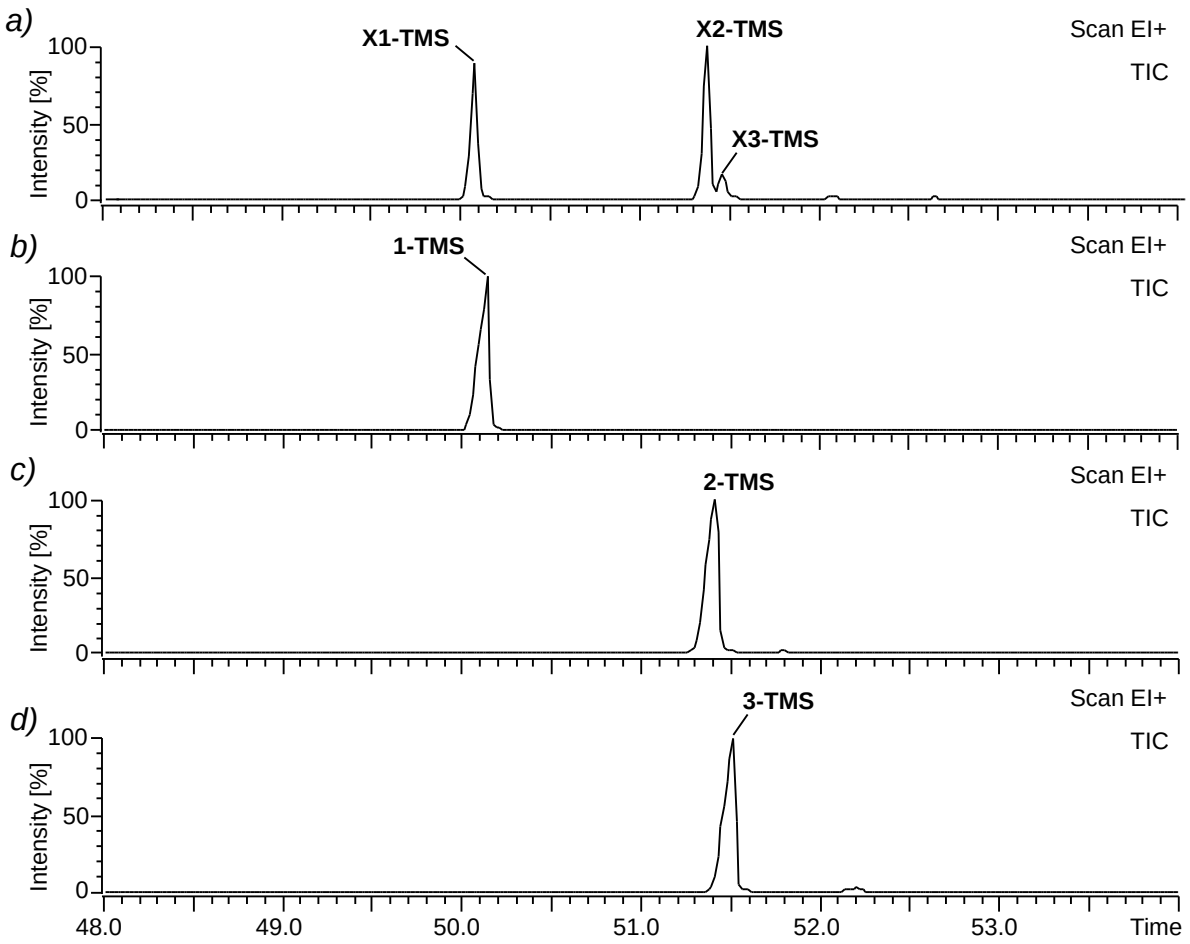



**Figure 4.** Sections of GC/MS profiles (EI) showing the silylated THX of *H. perforatum* seeds. *a)* THX fraction (polyamide MeOH extract). *b)* Reference compound: tetra-silylated 1,4,6,7-THX (**1-TMS**). *c)* tetra-silylated 1,2,6,7-THX (**2-TMS**). *d)* tetra-silylated 1,3,6,7-THX (**3-TMS**).

**Table 3.** *Kovats* retention indices (KRI) of derivatized tetrahydroxyxanthones (THX) from *H. perforatum*, calculated from GC/MS data, referenced to an *n*-alkane standard mixture (C27-C30) [30].

| Compound | Methylation with $CH_2N_2$ [a, b] | Silylation with BSTFA [a, c] |
|---|---|---|
| 1,4,6,7-THX (**X1**) | 2910.1 | 2813.8 |
| 1,2,6,7-THX (**X2**) | 2818.0 | 2888.5 |
| 1,3,6,7-THX (**X3**) | [d] | 2894.2 |

[a] Derivatization of all OH-groups. [b] A tri-methylated THX (peak **X0-Me**, see *Fig. 3*) at *m/z* 302 was also detected, revealing a KRI of 2764.4. [c] In accordance with the literature [25]. [d] Co-elution with **X1-Me**.

*Investigation of Morphology and Anatomy of* H. perforatum *and* H. tetrapterum *Seeds by SEM*

Scanning electron microscopy was used to investigate the morphology and anatomy of the tiny seeds of *H. perforatum* and *H. tetrapterum*. The seeds of both species are similar in shape and structure (*Fig. 5a-d*). They were oblong and rounded at both ends. The

dimensions of *H. perforatum* seeds were approximately 1 mm x 0.5 mm, while seeds of *H. tetrapterum* were comparatively smaller (0.6 mm x 0.3 mm). The surface of the testa (seed coat) exhibited a reticulate pattern (*Fig. 5b, d*), typical also for other *Hypericum* species [32]. Fractions of the seeds revealed an extremely thick two-layered testa around the embryo (*Fig. 5e*). The testa was built by a thin epidermal layer and a layer of thick-walled sclerenchyma cells (*Fig. 5f*). The imprints of the protruding anticlinal cell walls of the sclerenchyma cells contribute to the surface pattern of the testa (compare *Fig.5f*, arrow, and *Fig. 5b, d*, arrows). For *H. philonotis* a similarly built two-layered testa has been described [33], whereas *Skudlarz* found an additional thin layer between the epidermis and the thick sclerenchyma layer for seeds of *H. elegans* [32].

Recent biochemical studies have shown that the exodermis and endodermis of *H. perforatum* roots are sites of xanthone synthesis [34]. As known for exodermis and endodermis, the testa of a seed is not only a mechanical protective barrier, but also provides protection against microbial degradation. Xanthones act as phytoalexins to impair pathogen growth [23; 24] and show antifungal activities [35; 36]. The thick sclerenchyma layer of the testa is a strong protective layer for the seeds of *Hypericum*, but, besides lignins, it is also assumed to be a good storage place for phenolic compounds like THX. Thus, *Sánchez-Coronado* et al. demonstrated the phenolic character of the sclerenchyma layer of *H. philonotis* by vanilline HCl-staining [33]. About 32% of the seed weight is attributed to fatty oil [25] and the conspicuous compartments in the embryo were lipid droplets (not shown), whereas small phenolic-storing vacuoles cannot be excluded. Concerning the proportions of testa and embryo in these tiny seeds, the former may be a large reservoir for antimicrobial substances like xanthones. Interestingly, *Sánchesz-Corodado* et al. described that an acetone seed extract from *H. philonotis* partially inhibited seed germination [33]. It might be assumed that the xanthones are also responsible for the delayed germination of the seeds, which could be an important aspect of the plant's survival strategy. However, further investigations are required to verify this hypothesis.

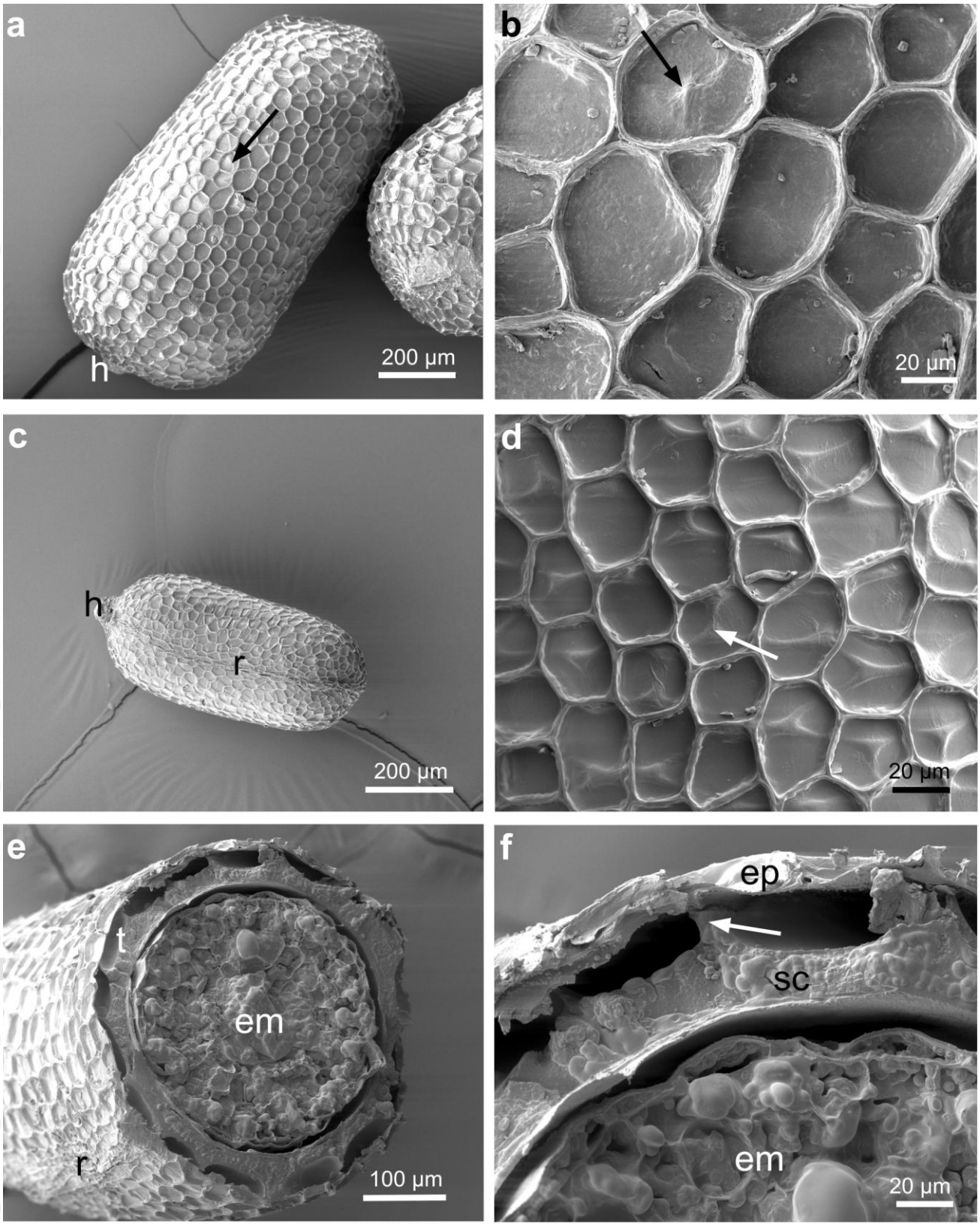



**Figure 5.** Scanning electron micrographs of dry seeds of *H. perforatum* and *H. tetrapterum*. *a)* Size and form of dry seeds of *H. perforatum*; hilum (h). *b)* Typical pattern of the testa of *H. perforatum*; imprint of the secondary layer of the testa (arrow). *c)* Size and form of a seed of *H. tetrapterum*; hilum (h), raphe (r). *d)* Typical pattern of the testa of *H. tetrapterum*. Imprint of the secondary layer of the testa (arrow). *e)* Cross fraction of a seed demonstrating the two-layered testa (t); embryo (em). *f)* Detail of *Fig. 5e*. The testa build by a thin epidermal layer (ep) and a layer of thick-walled sclerenchyma cells (sc) with protruding anticlinal walls (arrow); embryo (em).

## Conclusions

In summary, two novel tetrahydroxyxanthones **X1** and **X2** were extracted from seeds of *H. perforatum*, chromatographically purified and characterized via NMR as 1,4,6,7- and 1,2,6,7-THX (**1** and **2**, respectively). Structure assignment was confirmed by demonstrating chromatographic and spectrometric identity of synthesized **1** and **2** with the natural THX **X1** and **X2** from *Hypericum* seeds. Both THX were detected and quantified in seeds of *H. perforatum* and *H. tetrapterum*. To the best of our knowledge the THX isomers **1** and **2** were synthesized and detected as genuine plant metabolites for the first time. Since THX are known as pytoalexins, which protect plant tissue from oxidative damage and help to impair pathogen growth upon biotic stress, *e.g.* caused by microorganisms [23, 24], we assume that these molecules hold the same function in *Hypericum* seeds. This chemical defense system together with a strong mechanical barrier of lignified seed coats (testa), which has been visualized by SEM, is apparently an effective strategy to protect the seeds from decay during the long dormancy periods in the soil [33].

The different bioactivities of THX, in particular their anti-microbial and anti-oxidant properties [23; 34; 37], could make them interesting as bioactive ingredients for phytomedicinal, pharmaceutical or cosmetic applications. However, further investigations are needed to broaden our knowledge on their biological functions and to explore potential applications.

## Experimental Section

*General*

Boron tribromide ($BBr_3$), copper powder (Cu, <425 µm), 2,5-dimethoxybenzaldehyde and lithium diisopropylamide solution (LDA, 2 M) in THF/heptane/ethylbenzene were purchased from *Sigma-Aldrich* (*Merck*, Taufkirchen, Germany). 2-Bromo-4,5-dimethoxybenzoic acid was bought from *ChemPur* (Karlsruhe, Germany). 3,4-Dimethoxyphenol was obtained from *Alfa Aesar* (*Thermo Fisher(Kandel) GmbH*, Karlsruhe, Germany). For GC/MS analyses, the silylation reagent *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from *Fluka* (Buchs, Switzerland). *Sephadex® LH-20* was obtained from *GE Healthcare Bioscience* AB (Uppsala, Sweden), polyamide for column chromatography was from *Carl Roth GmbH + Co. KG* (Karlsruhe, Germany). Activated copper (Cu) bronze, *N*-methyl-*N*-nitroso-urea and 1,3,6,7-THX (**3**) were prepared according literature procedures [25; 38; 39], respectively.

*RP-HPLC-(DAD)/ESI-$MS^n$ Analyses*

Liquid chromatographic analyses were carried out on an *Agilent 1200* HPLC system (*Agilent Technologies Inc.*, Palo Alto, USA), equipped with a binary pump, a micro vacuum degasser, an autosampler, a thermostatic column compartment and a UV-VIS diode array detector. An *XSelect CSH®* C18 reversed-phase column (3.5 µm particle size, 100 x 2.1 mm i.d., *Waters Corporation*, Wexford, Ireland) was used for chromatographic separation at 25 °C at a flow rate of 0.34 ml/min. UV detection of the THX (*Fig. 2*) was performed at a wavelength of 260 nm. The mobile phase consisted of HCOOH/$H_2O$ 0.1/99.9 (*v/v*; eluent A) and HCOOH/MeCN 0.1/99.9 (*v/v*; mobile phase B). The injection volume of each sample was 10 µL, and the gradient used was as follows: 0 - 3 min, 0 - 5% *B*; 3 - 18 min, 5 - 20% *B*; 18 - 35 min, 20 - 30 % *B*; 35 - 50 min, 30 - 55% *B*; 50 - 60 min, 55 - 100% *B*; 60 - 65 min, 100 % *B*; 65 - 70 min, 100 - 0% *B*; 70 - 75 min, 0% *B*. The LC system was coupled to an *HCTultra* ion trap (*Bruker Daltonik GmbH*, Bremen, Germany) with an ESI source operating in the negative ionization mode and applying the following parameters: capillary voltage: +4000 V, dry gas ($N_2$) flow: 9.00 L/min with a capillary temperature of 365 °C; nebulizer pressure: 50 psi. Full scan mass spectra (mass range *m/z* 50 – 1000) of HPLC eluates were recorded during chromatographic separation yielding $[M\text{-}H]^-$ ions. To obtain further structural information, CID experiments were performed. $MS^n$ data were acquired in the auto MS/MS mode. The instruments were controlled by *Agilent Chemstation* and *EsquireControl* software (V7.1). Quantitation of the THX isomers (**X1**, **X2**, **X3**) was performed according to a previously published validated HPLC method. 1,3,6,7-THX (**3**) was used as a reference for external standard calibration [25].

*HR-ESI-HPLC-MS Analysis*

For determination of the exact molecular weight, a solution of synthesized **1** and **2** (0.01 mg/ml in MeOH) was applied to an *Agilent 1290* UHPLC system (*Agilent Technologies Inc.*, Palo Alto, USA) coupled to a *QExactive Plus Orbitrap* quadrupol-mass spectrometer (*Thermo Fisher Scientific*, Dreieich, Germany). Chromatographic separation of the analytes was performed on an *Acquity UPLC HSS T3*® C18-spherical silica column (100 Å pore size, 1.8 µm particle size, 150 x 2.1 mm i.d., *Waters Corporation*, Wexford, Ireland) at 40 °C. The mobile phase consisted of HCOOH/$H_2O$ 0.1/99.9 (*v/v*; eluent A) and HCOOH/MeCN 0.1/99.9 (*v/v*; eluent B). The injection volume of each sample was 10 µL, and the gradient used was as follows: 0 - 10 min, 10 -70 % *B*; 10 - 15 min, 70 - 90% *B*; 15 - 19 min, 90 % *B*; 19 - 20 min, 90 - 10% *B*; 20 - 26 min, 10% *B*, at a flow rate of 0.3 ml/min. Full scan mass spectra (ESI, mass range *m/z* 140 – 650) of HPLC eluates were recorded during chromatographic separation in the positive/negative ionization mode.

*GC/MS Analyses*

GC/MS analyses were performed with a *PerkinElmer Clarus 500* gas chromatograph with split injection (split ratio 30 : 1, injection volume 1.0 µL), coupled to a single quadrupole mass detector. The column used was a *Zebron ZB-5MS* capillary column (60 m x 0.25 mm i. d. x 0.25 µm film thickness, 5% phenylpolysiloxane and 95% dimethylpolysiloxane coating; *Phenomenex*, Torrance, USA). Carrier gas: helium at a flow rate of 1 ml/min. The injector used was a PSS (temperature-programmed split/splitless injector temperature: 250 °C). The temperature program for the column oven was 100 to 320 °C with a linear gradient of 4 °C/min and a final hold time of 30 min. The mass spectrometer was run in electron ionization (EI) mode (70 eV). The software *Turbomass* (v.5.4.2, *PerkinElmer Inc.*, MA, USA) was used for data acquisition and processing.

*NMR Spectroscopy*

NMR spectra were recorded in DMSO-$d_6$ at 500 or 600 ($^1$H) and 125 or 150 MHz ($^{13}$C), respectively, using a 500 MHz *Varian Inova* and a 600 MHz *Bruker Avance III HD NMR* spectrometer equipped with a *BBO Prodigy* cryo-probe. Chemical shifts are reported in $\delta$[ppm] and referenced to residual (non-deuterated) solvent signals of DMSO-$d_6$ ($^1$H: $\delta$2.50; $^{13}$C: $\delta$39.51 ppm). $^{13}$C-NMR signal assignment of the novel compounds was based on 2D-heteronuclear NMR experiments (*g*HSQC, *g*HMBC and PIP-HSQMBC [40]). For evaluation of NMR spectra the program *SpinWorks 3.1.7*. (Copyright® 2010, *K. Marat*, University of Manitoba, USA) was used.

*Scanning Electron Microscopy (SEM)*

Dry seeds and variable seed fractions of *H. perforatum* and *H. tetrapterum* were mounted on adhesive carbon tabs on Al-stubs, sputter-coated with gold-palladium (*SCD 040*, *Balzer Union*, Wallruf, Germany) and investigated using a scanning electron microscope *DSM 940* (*Zeiss*, Oberkochen, Germany) at 5 kV. The fractions were obtained with the aid of a strong razor blade under a binocular microscope.

*Plant Material*

Seeds of *H. perforatum* L. and *H. tetrapterum* FR. were acquired from *Jelitto Perenial Seeds GmbH* (Schwarmstedt, Germany). Both species were identified by Prof. Dr. *O. Spring* and voucher specimens (voucher number: HOH-016142 (*H. perforatum*); HOH-016143 (*H. tetrapterum*)) were deposited with the herbarium of the Department of Botany at Hohenheim University (Germany).

*Extraction of* H. perforatum *Seeds, Chromatographic Purification of the THX*

Seeds of *H. perforatum* (2 x 100.0 g) were immersed in two batches with $CH_2Cl_2$ (2 x 700 ml), and each batch was minced by *Ultra-Turrax* treatment (3 min; 21000 rpm, *IKA Werke GmbH & Co. KG*, Staufen, Germany), under external ice cooling. After maceration over night at 4 °C the seeds were filtered off over *Celite* by vacuum suction and extracted a second time in the same manner. An oil fraction (63.51 g; 31.7% of the seed weight) was recovered from the combined $CH_2Cl_2$ extracts by vacuum rotoevaporation of the solvent. Subsequently, the degreased seeds were extracted twice with MeOH (2 x 700 ml each), filtered off and MeOH was removed *in vacuo* to yield 7.26 g (3.63 % of the seed weight) of a crude extract. The latter was suspended in water (1.2 l) by *Ultra-Turrax* treatment, and the slurry was stirred with polyamide (150 g) for 30 min using a blade agitator. After filtration over a nylon sieve (mesh size <125 µm) the filtrate was treated a second time with polyamide (50 g). Subsequently, the polyamide was washed with water (6 x 1 l) and compounds bound onto the sorbent were eluted by stirring with MeOH (4 x 1 l, 15 min at a time). The solvent was distilled off from the combined MeOH extracts by vacuum rotoevaporation to yield a solid orange residue (1.76 g). A solution of the latter (0.61 g in 5 ml MeOH) was loaded onto a vacuum liquid chromatography column (VLC, 100 g silica 60 G, preconditioned with 300 ml $CH_2Cl_2$) and the THX were eluted utilizing a $CH_2Cl_2$/MeOH gradient (from 99:1 to 90:10, *v/v*). The fractionation was monitored by TLC (silica 60 G; $CH_2Cl_2$/MeOH, 16:4, *v/v*). Three Fractions (F3 - F5), containing the THX isomers, were unified, and the solvent was removed *in vacuo* (total amount of the THX-enriched fraction: 0.0265 g). An aliquot of this fraction was investigated by NMR and GC/MS (after BSTFA-silylation and methylation with $CH_2N_2$, respectively).

*Methylation and Silylation of the THX for GC/MS Analysis*

*Methylation*. The THX enriched fraction (20 mg, see above) was dissolved in a MeOH/$H_2O$ mixture (9:1, v/v, 10 ml) and treated with a $CH_2N_2$ solution in $Et_2O$, which was prepared from *N*-methyl-*N*-nitroso-urea (4.00 g) and aqueous KOH solution (40%, w/v, 10 ml) in $Et_2O$ (48 ml), under external cooling (ice/NaCl mixture) [39]. After stirring for 3 h at r.t., $Et_2O$ was gently evaporated under a stream of $N_2$ and the remaining solvent removed by rotoevaporation. For complete conversion the residue was treated for a second time with $CH_2N_2$. After solvent evaporation the residue was dissolved in $CHCl_3$ (5 ml) and investigated by GC/MS. *Silylation*. Silylation was performed according to a previously published procedure [25]. *Kovats* retention indices of the analytes were determined in compliance with a literature protocol [30] and referenced to an *n*-alkane standard mixture ($C_{27}$-$C_{30}$, *Fig. 3b*).

*Synthesis of the Reference Compound 1,2,6,7-Tetrahydroxyxanthone* (= 1,2,6,7-Tetrahydroxy-9H-xanthen-9-one; **2**).

**2-Bromo-4,5-dimethoxybenzoic acid methylester** (**4**). A solution of 2-bromo-4,5-dimethoxybenzoic acid (6.00 g, 22.98 mmol), MeOH (200 ml) and $H_2SO_4$ (97%, 2 ml) was refluxed for 6 h. Afterwards, the mixture was treated with a sat. aqueous $NaHCO_3$ solution (1%, *w/v*, 100 ml) and water (200 ml) and extracted with $Et_2O$ (4 x 200 ml). The combined $Et_2O$ extracts were dried ($Na_2SO_4$), the desiccant filtered off, washed with $Et_2O$ (3 x 50 ml) and the solvent was removed by vacuum rotoevaporation. The yielded **4** was dried at 40 °C *in vacuo* (at <2 mbar). Yield: 5.65 g (89.5% of the theory). White crystals. Mp. 82 - 83 °C (lit. M.p., from MeOH, 87-89 °C) [41]. UV/VIS (MeCN): 222 (4.40), 261 (3.94), 294 (3.56). GC/MS purity (70 eV): >99.4% ($t_R$ 29.0 min): *m/z* 276, 274 (99, 100, $M(^{81}Br/^{79}Br)^+$), 261 (11), 259 (11), 245 (82), 243 (84), 215 (7), 199 (15), 157 (14), 137 (13), 108 (16), 93 (22), 77 (21).

**2-(3,4-Dimethoxyphenoxy)-4,5-dimethoxybenzoic acid methylester** (**8**). A mixture of **4** (5.60 g, 20.36 mmol), 3,4-dimethoxyphenol (**6**; 3.14 g, 20.37 mmol), Cu bronze (2.69 g, 42.33 mmol), $Na_2CO_3$ (4.49 g, 42.36 mmol) and dried pyridine (84 ml) was stirred and refluxed under $N_2$ atmosphere (bath temperature 250 °C) for 24 h. Hereinafter, pyridine was distilled off by rotoevaporation. The yielded black tar residue (21.97 g) was dissolved in AcOEt (100 ml) and filtered

over *Celite*, which was washed with *n*-hexane/AcOEt (1/1, *v/v*, 100 ml). From the combined filtrates the solvent was removed *in vacuo* to yield a dark tar residue, vacuum dried at 45 °C for 30 min (at 3 mbar). From the residue (6.36 g) the pure **8** was obtained by repeated vacuum liquid chromatography (VLC) on silica 60 G (2 x 100 g SiO2), utilizing an *n*-hexane/AcOEt gradient (from 100/0 to 60/40, v/v). The fractions containing **8** (detected by TLC) were unified, the solvent was removed by rotoevaporation, and the product was dried *in vacuo*. Yield: 2.80 g (39.5 % of the theory). White solid. M.p. 108 °C. $R_f$ ($SiO_2$; *n*-hexane/AcOEt 1:1(*v/v*)) 0.35. UV/VIS (MeCN): 222 (4.50), 257 (4.11), 290 (3.88). GC/MS purity (70 eV): >99.9 % ($t_R$ 45.2 min): *m/z* 348 (59, $M^+$), 333 (1, $[M\text{-}CH_3]^+$), 317 (4, $[M\text{-}OCH_3]^+$), 301 (3), 273 (3), 258 (6), 243 (3), 231 (3), 215 (3), 187 (2), 165 (3), 153 (100), 137 (6), 125 (22), 107 (6), 93 (5), 79 (13). $^1$H-NMR (500 MHz): 7.35 (*s*, H-(C6)); 6.85 (*d*, *J*=8.8, H-(C5′)); 6.65 (*s*, H-(C3)); 6.64 (*s*, H-(C2′)); 6.25 (*dd*, *J*=2.7, 8.7, H-(C6′); 3.80 (*s*, H-(C7)); 3.73 (*s*, H-(C8)); 3.72 (*s*, H-(C8′); 3.70 (*s*, H-(C7′); 3.68 (*s*, H-(C10)). $^{13}$C-NMR (125 MHz): 164.80 (C(9)); 153.25 (C(4)); 152.13 (C(1′)); 150.40 (C(2)); 149.68 (C(4′)); 144.83 (C(5)); 144.30 (C(3′)); 113.64 (C(1)); 113.02 (C(6)); 112.47 (C(5′)); 107.19 (C(6′)); 105.43 (C(3)); 102.65 (C(2′)); 55.92, 55.91 (C(7′), C(8)); 55.85 (C(7)); 55.59 (C(8′)); 51.75 (C(10)).

**2-(3,4-Dimethoxyphenoxy)-4,5-dimethoxybenzoic acid** (**9**). A solution of **8** (2.02 g, 5.80 mmol) in THF/MeOH (1:1, *v/v*, 50 ml) was treated under external ice cooling ($N_2$ atmosphere) with an aqueous NaOH solution (5 M, 10 ml) and stirred at r.t. for 24 h. Then, water was added (200 ml) and the solution washed with $CH_2Cl_2$ (250 ml), the aqueous layer was acidified with HCl (37%, 25 ml) and extracted with $CH_2Cl_2$ (2x 250 ml). After drying ($Na_2SO_4$), the solvent was removed from the $CH_2Cl_2$ extract by vacuum rotary evaporation to give crude **9** (1.75 g, 90.7% of the theory), which was used for the next reaction step without further purification. Creme-colored solid. M.p. 194.7 °C (after recrystallization from *n*-hexane/AcOEt). $R_f$ ($SiO_2$; toluene/AcOEt/HCOOH, 10:9:1 (*v/v/v*)) 0.45. UV/VIS (MeCN): 223 (4.49), 258 (4.12), 289 (3.83). GC/MS purity (70 eV): >99 % (TMSi derivative, $t_R$ 45.8 min): *m/z* 406 (18, $M^+$), 391 (6, $[M\text{-}CH_3]^+$), 317 (4), 301 (4), 273 (3), 253 (100), 211 (11), 209 (13), 165 (4), 137 (4), 107 (4), 75 (10), 73 (40, $[Si(CH_3)_3]^+$). $^1$H-NMR (500 MHz): 7.36 (*s*, H-(C6)); 6.84 (*d*, *J*=8.8, H-(C5′)); 6.63 (d, *J*=2.6, H-(C2′)); 6.62 (*s*, H-(C3)); 6.23 (*dd*, *J*=2.7, 8.7, H-(C6′); 3.79 (*s*, H-(C7)); 3.72 (*s*, H-(C8)); 3.71 (*s*, H-(C8′); 3.69 (*s*, H-(C7′). $^{13}$C-NMR (125 MHz): 165.91 (C(9)); 152.91 (C(4)); 152.39 (C(1′)); 150.13 (C(2)); 149.67 (C(4′)); 144.79 (C(5)); 144.15 (C(3′)); 115.07 (C(1)); 113.33 (C(6)); 112.56 (C(5′)); 107.07 (C(6′)); 105.48 (C(3)); 102.63 (C(2′)); 55.98 (C(7′)); 55.87 (C(8)); 55.78 (C(7)); 55.57 (C(8′)).

**1,2,6,7-Tetramethoxyxanthone** (= **1,2,6,7-Tetramethoxy-9H-xanthen-9-one**; **10**). Compound **9** (1.75 g, 5.24 mmol), dissolved in 100 ml dry THF, was treated under $N_2$ and external cooling (NaCl/ice mixture) with a LDA solution in THF (2 M, 6.0 ml). Afterwards, the mixture was stirred for 1 h at 0 °C and further 75 min at r. t. Then, the reaction was quenched by adding ice, the mixture acidified with HCl (5 %, *w/w*, 100 ml) and extracted with $CH_2Cl_2$ (4 x 100 ml). After drying ($Na_2SO_4$) the solvent was removed by rotorary evaporation. Because of incomplete conversion of **9** (detected by GC/MS), the obtained residue was dissolved again in THF (100 ml), treated for a second time with LDA solution (2 M, 12 ml) and treated in the same manner (see above). The yielded crude product (1.81 g) was purified by VLC on silica 60 G (100 g $SiO_2$), utilizing an *n*-hexane/AcOEt gradient (from 100/0 to 30/70, *v/v*). The fractions containing **10** were unified, the solvent was removed by rotoevaporation, and the product was dried *in vacuo*. Yield: 0.76 g (46.4% of the theory). Creme-colored solid. M.p. 174 °C. $R_f$ ($SiO_2$; *n*-hexane/AcOEt 15:1 (*v/v*)) 0.40. UV/VIS (MeCN): 246 (4.57), 276 (sh, 4.13), 318 (4.01), 362 (3.92). GC/MS purity (70 eV): >99.5 % ($t_R$ 49.9 min): *m/z* 316 (65, $M^+$), 301 (100), 287 (32), 273 (32), 257 (18), 243 (11), 229 (20), 215 (10), 200 (6), 187 (5), 171 (5), 150 (40), 137 (9), 128 (8), 106 (5), 78 (12). $^1$H-NMR (600 MHz): 7.56 (*d*, *J* = 9.3, H-(C3)); 7.44 (*s*, H-(C8)); 7.32 (*d*, *J* = 9.2, H-(C4)); 7.08 (*s*, H-(C5)); 3.92 (*s*, H-(C12)); 3.86 (*s*, H-(C11)); 3.85 (*s*, H-(C13)); 3.80 (*s*, H-(C10)). $^{13}$C-NMR (150 MHz): 173.94 (C(9)); 155.12 (C(6)); 150.85 (C(5a)); 150.38 (C(4a)); 148.79 (C(2)); 147.43 (C(1)); 146.33 (C(7)); 119.87 (C(3)); 116.00 (C(1a)); 114.22 (C(8a)); 112.97 (C(4)); 104.84 (C(8)); 99.68 (C(5)); 60.99 (C(10)); 56.63 (C(11)); 56.33 (C(12)); 55.70 (C(13)). C-Atom assignment by *g*HSQC, *g*HMBC and PIP-HSQMBC[40] (*Fig. 6*).

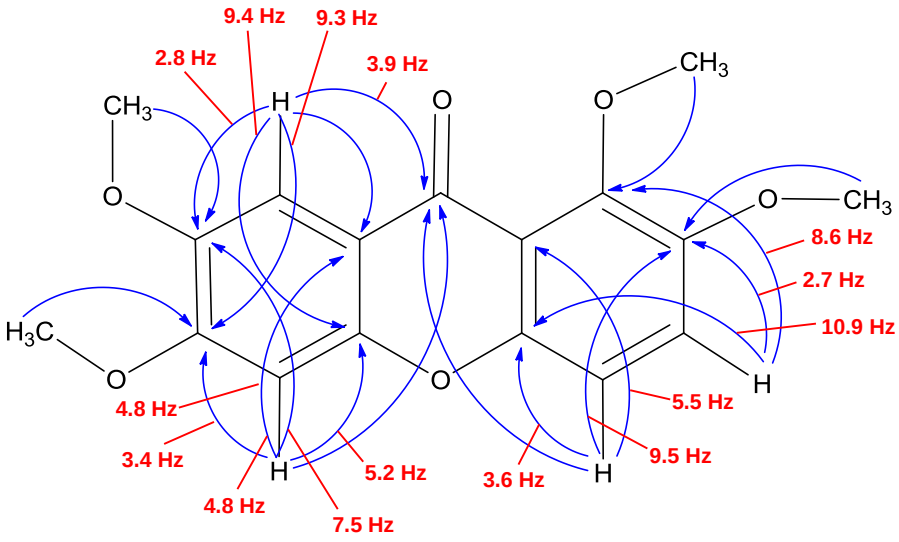



**Figure 6.** $^{1}H$-$^{13}C$ long range couplings for **10** (blue arrows: HMBC correlations; red: PIP-HSQMBC correlations, $^{1}H$-$^{13}C$ long range $J_{CH}$).

**1,2,6,7-Tetrahydroxyxanthone** (= **1,2,6,7-Tetrahydroxy-9H-xanthen-9-one**; **2**). A solution of **10** (0.177 g, 0.56 mmol) in $CH_2Cl_2$ (52 ml), externally cooled with an ice/NaCl-mixture, was treated under $N_2$ atmosphere with $BBr_3$ (0.7 ml, 1.85 g, 7.38 mmol), stirred for 4 h at 0 °C and further at r.t. (3 h). Subsequently, the reaction mixture was poured on ice and extracted with AcOEt (3 x 200 ml). The combined organic phases were dried ($Na_2SO_4$) and the solvent removed by vacuum rotoevaporation to yield an orange colored residue (0.278 g). The latter was purified by VLC on silica 60 G (30 g $SiO_2$), applying a $CH_2Cl_2$/MeOH linear gradient (from 100/0 to 70/30, *v/v*) to yield the TLC-pure **2** (0.10 g, 68.6 % of the theory). The latter was further purified on *Sephadex® LH-20* by use of a $CH_2Cl_2$/MeOH linear gradient (from 100/0 to 80/20, *v/v*) to give the NMR-pure **2** (0.067 g, 46.2 % of the theory) as a lemon-yellow solid. $R_f$ ($SiO_2$; $CHCl_3$/AcOH/$H_2O$ 10:8.4:1.6 (*v/v/v*)) 0.57. UV/VIS (MeCN): 236 (4.32), 249 (4.28), 265 (4.22), 271 (*sh*, 4.21), 290 (4.07), 326 (3.95), 388 (3.78). GC/MS purity (70 eV): 98 % (TMSi derivative, $t_R$ 51.4 min): *m/z* 548 (2, $M^+$), 534 (45), 533 (90), 517 (1), 475 (1), 460 (3), 445 (16), 415 (3), 373 (6), 343 (1), 259 (2), 147 (3), 73 (100, $[Si(CH_3)_3]^+$). HR-MS-ESI$^-$ *m/z* 259.02429 $[M\text{-}H]^-$ (calcd. for $C_{13}H_7O_6$, 259.02426). HR-MS-ESI$^+$ *m/z* 261.03909 $[M+H]^+$ (calcd. for $C_{13}H_9O_6$, 261.03936). NMR data: see *Tab. 1*.

*Synthesis of the Reference Compound 1,4,6,7-Tetrahydroxyxanthone* (= 1,4,6,7-Tetrahydroxy-9H-xanthen-9-one; **1**).

**2,5-Dimethoxyphenol** (**5**). Starting from 2,5-dimethoxybenzaldehyde, compound **5** was obtained with 57.6 % yield as a light brown oil, via *Dakin* reaction, according to a literature procedure [42]. $R_f$ ($SiO_2$; *n*-hexane/AcOEt 1:1 (*v/v*)) 0.53. GC/MS purity (70 eV): 99 % ($t_R$ 14.7 min): *m/z* 154 (68, $M^+$), 139 (100), 125 (3), 111 (62), 96 (11), 79 (8), 69 (11).

**2-(2,5-Dimethoxyphenoxy)-4,5-dimethoxybenzoic acid methylester** (**7**). The synthesis of **7** followed an analogous procedure as described for the synthesis of **8** (see above), starting from **4** (5.00 g, 18.18 mmol) and **5** (2.87 g, 18.62 mmol). Yield: 2.68 g (42.4% of the theory). Slightly pink syrup. $R_f$ ($SiO_2$; *n*-hexane/AcOEt 1:1 (*v/v*)) 0.35. UV/VIS (MeCN): 222 (4.72), 258 (4.25), 293 (4.08). GC/MS purity (70 eV): >99.8 % ($t_R$ 44.7 min): *m/z* 348 (80, $M^+$), 333 (4, $[M\text{-}CH_3]^+$), 317 (6, $[M\text{-}OCH_3]^+$), 301 (2), 274 (15, $[M\text{-}COOCH_3\text{-}CH_3]^+$), 258 (8), 243 (5), 209 (11), 179 (3), 168 (4), 153 (100), 137 (8), 125 (23), 107 (12), 79 (14), 77 (13). $^{1}H$-NMR (600 MHz): 7.36 (*s*, H-(C6)); 7.03 (*d*, *J*=8.9, H-(C5')); 6.61 (*dd*, *J*=2.9, 8.9, H-(C4')); 6.54 (*s*, H-(C3)); 6.22 (*d*, *J*=2.9, H-(C2')). $^{13}C$-NMR (150 MHz): 164.65 (C(9)); 153.61 (C(3')); 153.30 (C(4)); 150.35 (C(2)); 147.19 (C(1')); 144.64 (C(5)); 143.88 (C(6')); 114.47 (C(5')); 113.13 (C(6)); 112.85 (C(1)); 107.09 (C(4')); 104.65 (C(2')); 104.29 (C(3)); 56.54 (C(8')); 55.88, 55.86 (C(7),C(8)); 55.37 (C(7')); 51.70 (C(10)).

**1,4,6,7-Tetramethoxyxanthone** (= **1,4,6,7-Tetramethoxy-9H-xanthen-9-one**; **11**).The synthesis of **11** was realized by directly starting from the methylester **7**. For this purpose, a solution of **7** (2.68 g, 7.70 mmol) in dry THF (150 ml) was treated under $N_2$ atmosphere by external cooling (ice/NaCl-mixture) by dropwise addition of a LDA solution in THF (2 M, 23.1 ml, resp. 4.95 g LDA, 46.21 mmol). Then, the reaction mixture was stirred at 0 °C (30 min) and at r.t. (4 h). Thereafter, the reaction mixture was poured on ice, acidified with HCl (5 %, *w/w*, 150 ml) and extracted with $CH_2Cl_2$ (4 x 150 ml). After drying ($Na_2SO_4$) the solvent was removed by rotary evaporation. The obtained dark residue (3.24 g) was purified by repeated VLC on silica 60 G (100 g $SiO_2$), utilizing a linear *n*-hexane/AcOEt gradient (from 100/0 to 30/70, *v/v*). The fractions containing **11** were combined, the solvent was removed by rotoevaporation and the product was dried *in vacuo*. Yield: 0.27 g (11.1 % of the theory). Pink-colored crystals. $R_f$ ($SiO_2$; $CH_2Cl_2$/MeOH 19:1 (v/v)) 0.50. UV/VIS (MeCN): 204 (4.44), 244 (4.36), 250 (*sh*, 4.38), 271 (4.28), 363 (3.95). GC/MS purity (70 eV): 99 %. ($t_R$ 51.8 min): *m/z* 316 (100, $M^+$), 301 (80, $[M\text{-}CH_3]^+$), 287 (72), 283 (19), 273 (41, $[M\text{-}CH_3\text{-}CO]^+$), 272 (34), 257 (8), 243 (18), 228 (12), 215 (11), 200 (8), 187 (8), 165 (7), 158 (13), 143 (8), 137 (14), 121 (7), 115 (7), 93 (6), 77 (13). $^1$H-NMR (600 MHz): 7.42 (*s*, H-(C8)); 7.38 (*d*, *J* = 9.0, H-(C3)); 7.16 (*s*, H-(C5)); 6.86 (*d*, *J* = 9.0, H-(C2)); 3.93 (*s*, H-(C12)); 3.91 (*s*, H-(C11)); 3.85 (*s*, H-(C13)); 3.82 (*s*, H-(C10)). $^{13}$C-NMR (150 MHz): 173.71 (C(9)); 154.92 (C(6)); 152.77 (C(1)); 150.30 (C(5a)); 146.96 (C(4a)); 146.60 (C(7)); 141.83 (C(4)); 116.31 (C(3)); 115.03 (C(8a)); 112.21 (C(1a)); 105.35 (C(2)); 104.80 (C(8)); 100.00 (C(5)); 56.44 (C(12)); 56.37 (C(11)); 56.34 (C(10)); 55.74 (C(13)). C-Atom assignment by *g*HSQC, *g*HMBC and PIP-HSQMBC[40] (*Fig. 7*).

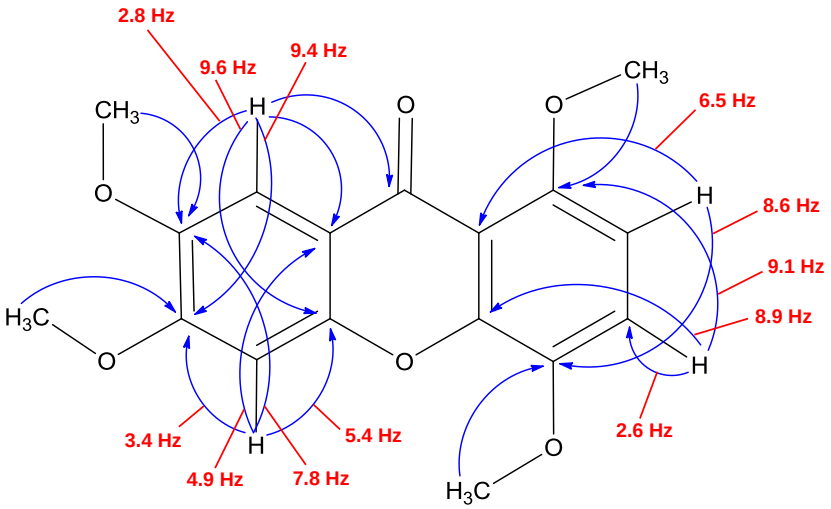



**Figure 7.** $^1$H-$^{13}$C long range couplings for **11** (blue arrows: HMBC correlations; red: PIP-HSQMBC correlations, $^1$H-$^{13}$C long range $J_{CH}$).

**1,4,6,7-Tetrahydroxyxanthone** (**= 1,4,6,7-Tetrahydroxy-9H-xanthen-9-one**; **1**). 1,4,6,7-THX (**1**) was prepared according to a protocol similar to that utilized for the synthesis of 1,2,6,7-THX (**2**, see above), though starting from **11** (0.27 g, 0.86 mmol). Yield: 0.082 g (37.2% of the theory). Beige-colored powder. $R_f$ ($SiO_2$; $CHCl_3$/AcOH/$H_2O$ 10:8.4:1.6 (*v/v/v*)) 0.52. UV/VIS (MeCN): 224 (4.26), 256 (4.26), 279 (4.31), 321 (*sh*, 3.67), 389 (3.87). GC/MS purity (70 eV): 99.9 % (TMSi derivative, $t_R$ 50.1 min): *m/z* 548 (1, $M^+$), 534 (45), 533 (94), 517 (1), 476 (2), 460 (3), 445 (17), 415 (3), 373 (7), 343 (2), 259 (1), 147 (2), 73 (100, $[Si(CH_3)_3]^+$). HR-MS-ESI$^-$ $[M\text{-}H]^-$ *m/z* 259.02499 (calcd. for $C_{13}H_7O_6$, 259.02426). HR-MS-ESI$^+$ $[M+H]^+$ *m/z* 261.03958 (calcd. for $C_{13}H_9O_6$, 261.03936). NMR data: see *Tab. 1*.

*Synthesis of the Reference Compound* 1,3,6,7-Tetramethoxyxanthone (= 1,3,6,7-Tetramethoxy-9H-xanthen-9-one; **12**).

Compound **12** *w*as synthesized from 1,3,6,7-THX (**3**) by treatment with $CH_2N_2$/MeOH/$Et_2O$ (see above) and used for GC/MS comparison without further purification. GC/MS (70 eV; $t_R$ 51.6 min): *m/z* 316 (100, $M^+$), 315 (48), 299 (15), 287 (51), 285 (24), 271 (27), 270 (38), 255 (8), 243 (22), 215 (10), 200 (21), 185 (10), 158 (9), 144 (15), 129 (9), 106 (7), 77 (8).

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## Author Contribution Statement

Design of the study: *P. L., D. R. K.;* preparation of extracts, synthesis of THX: *P. L.;* data acquisition: *P. L., M. Bu., M. H., M. Be.;* NMR data interpretation: *P. L., J. C.;* acquisition and interpretation of SEM data: *A. H.;* evaluation of the data and preparation of the manuscript: *P. L., A. H., M. H., D. R. K.,* and *F. C. S.*

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