Antibacterial scalarane from *Doriprismatica stellata* nudibranchs (Gastropoda, Nudibranchia), egg ribbons, and their dietary sponge *Spongia cf. agaricina* (Demospongiae, Dictyoceratida)

Cora Hertzer¹, Stefan Kehraus¹, Nils Böhringer²,³, Fontje Kaligis⁴,§, Robert Bara⁴, Dirk Erpenbeck⁵,⁶, Gert Wörheide⁵,⁶,⁷, Till F. Schäberle²,³, Heike Wägele⁸ and Gabriele M. König*¹,¶

**Abstract**

Investigations on the biochemical relationship between *Doriprismatica stellata* (Chromodorididae, Doridoidea) nudibranchs, their egg ribbons, and the associated dietary sponge *Spongia cf. agaricina* (Demospongiae, Porifera) led to the isolation of the structurally new scalarane-type sesterterpene 12-deacetoxy-4-demethyl-11,24-diacetoxy-3,4-methylenedeoxoscalarin, with an unprecedented position of the cyclopropane ring annelated to the ring A. Unlike other scalaranes, which are most often functionalized at
C-12 of ring C, it bears two acetoxy groups at C-11 and C-24 instead. The compound was present in all three samples, supporting the dietary relationship between chromodorid nudibranchs of the genus Doriprismatica and scalarane-containing dictyoceratid sponges of the Spongiidae family. The results also indicate that D. stellata passes the scalarane metabolite on to its egg ribbons, most likely for protective purposes. The scalarane showed antibacterial activity against the Gram-positive bacteria Arthrobacter crystallopoietes (DSM 20117) and Bacillus megaterium (DSM 32).

Introduction

In habitats with intense competition and feeding pressure, such as coral reefs, sessile or slow-moving organisms commonly defend themselves with toxic or deterrent molecules [1-8]. Sponges (Porifera), for example, represent one of the main sources of marine bioactive natural products, due to their impressive chemical armory [4]. These specialized metabolites can be produced either by the sponge itself or by associated microbial symbionts [9-16]. Their production is assumed to be useful against numerous environmental stress factors, such as predation, pathogens, overgrowth by fouling organisms, or competition for space [4,10,15,17].

Though defensive metabolites are effective against most predators, some also attract nudibranchs of the family Chromodorididae (Gastropoda, Mollusca). These colorful, shell-less sea slugs are specialized to live and feed on noxious demosponges (Demospongiae, Porifera). They evolved the ability to sequester, accumulate, and store spongian metabolites to their own advantage [2,5,9,18-33]. Besides, specific metabolites can be passed on from the sea slugs to their similarly conspicuous and physically defenceless eggs. This has been shown exemplarily for the egg ribbons of certain nudipleuran taxa, such as Hexabranchus sanguineus [17], Pleurobranchaea maculata [34], Cadlina luteomarginata [35], and the two Dendrodoris species D. grandiflora and D. limbata [36]. The passing on of special metabolites from sea slugs to their egg ribbons suggests an additional biological role in the reproductive cycle or as protection of the eggs against predation or fouling.

Chemotaxonomic approaches have shown that chromodorid nudibranchs of the genera Chromodoris, Doriprismatica, Felimare, Felimida, Glossodoris/Casella, and Goniobranchus sequester and reuse spongian-type furanoterpenoids, diterpenoids, and sesquiterpenoids, or scalarane-type sesquiterpenoids and sesterterpenoids from their sponge prey [23,37-45]. However, confusion in the chemotaxonomy of Chromodorididae arose by multiple changes in the species names, including splitting and synonymizations, and the inclusion of species that have since been discovered to be members of other genera. Additionally, a splitting of generic groups into several genera and resurrection of old names increased the confusion [39,42,46-49]. To classify specialized metabolites in the Chromodorididae in a meaningful way, a solid understanding of their taxonomy, biology, and prey is essential.

Members of Glossodoris/Casella and Doriprismatica represent such a case of complex systematic challenges and complicated taxonomic histories [49]. Previous work on Doriprismatica (former Glossodoris) sedna [39] and Doriprismatica (former Glossodoris or Casella) atromarginata [38,41,44,45,50], reported the isolation of scalaranes, homoscalaranes, norscalaranes, spongian diterpenoids and furanoditerpenoids. A dietary origin of these molecules was inferred and attributed to dictyoceratid sponges of the genera Hyrtios and Carte-riospongia (Thorectidae), as well as Hyattella and Spongia (Spongidae). A geographical variation was described between D. atromarginata populations from Sri Lanka and Australia, containing furanoditerpenes, and a D. atromarginata population from India, containing scalarane sesterterpenes as a consequence of sponge prey availability [41]. The isolated metabolites showed various biological activities, such as cytotoxicity, antimicrobial, antiviral and antitumor activities, inhibition of transactivation for the farnesoid X receptor, inhibition of mammalian phospholipase A₂, and ichthyotoxicity against the mosquitofish Gambusia affinis [28,29,39,51-56]. Furthermore, a Vietnamese collection of D. atromarginata was found on the gorgonian Menella woodin (Plexauridae, Alcyonacea). Instead of spongian- or scalarane-type metabolites, they contained steroidal compounds, presumably sequestered from M. woodin [57].

Here, we report the first investigation on the biochemical relationship between Doriprismatica (former Glossodoris) stellata (Chromodorididae, Doridina) of the Indo-West Pacific (Figure 1), their egg ribbons, and the associated dietary sponge, identified as Spongia cf. agaricina (Spongidae, Demospongiae). We describe the structure elucidation of the new scalarane sesterterpene 12-deacetoxy-4-demethyl-11,24-diaceoxy-3,4-methylenedeoxyoscalarin (Figure 2), isolated from all our Doriprismatica stellata nudibranch, egg ribbon and Spongia cf. agaricina samples (Figure 3). It is the first scalarane sesterterpene reported with a cyclopropane ring bridging the carbons C-3, C-22 and C-4 in ring A, and an acetoxy group at C-11 instead of C-12 in ring C (Figure 2). All ethyl acetate
Results

Chemical investigation on *Doriprismatica stellata* nudibranchs, egg ribbons and *Spongia* cf. *agaricina*

The new molecule was isolated as a white amorphous solid from *D. stellata* nudibranchs (11 mg, 0.3% wet weight). Specific optical rotation was measured in chloroform (c = 0.6), giving $\left[\alpha\right]_D +40.5$. The molecular formula C$_{29}$H$_{42}$O$_6$ was established based on $^{13}$C NMR data and HRAPCI-MS measurements, yielding m/z 487.3054 [M + H]$^+$ (Supporting Information File 1). The double bond equivalent (DBE) was calculated to be nine and together with the $^{13}$C NMR data, giving evidence for one C–C and two C–O double bonds, thus suggested a structure.
The planar structure of 12-deacetoxy-4-demethyl-11,24-diacetoxy-3,4-methylenedioxoscalarin was established by extensive 1D and 2D NMR experiments ($^1$H, $^{13}$C, $^1$H,$^1$H-COSY, DEPT, HSQC and HMBC, see Table 1, Figure S8, Supporting Informa-

### Table 1: NMR spectroscopic data of 12-deacetoxy-4-demethyl-11,24-diacetoxy-3,4-methylenedioxoscalarin (CDCl₃).

| C     | δ_H (mult. J in Hz) | δ_C | COSY          | HMBC                        | NOESY                        |
|-------|---------------------|-----|---------------|-----------------------------|------------------------------|
| 1β    | 1.73, m             | 35.3, CH₂ | H-1α, H-2αβ  | C-2, C-3, C-5, C-9, C-10,  | H-1α, H-11                   |
| 1α    | 0.53, m             | 23.9, CH₂ | H-1β, H-2αβ  | C-2, C-3, C-5, C-9, C-10,  | H-1β, H-22b                  |
| 2β    | 1.96, m             | 19.0, CH₂ | H-3, H-1αβ, H-2α | C-11, C-13, C-15, C-18, C-24-OAc | H-11, C-12, C-14, C-18, C-24 |
| 2α    | 1.70, m             | 17.9, CH₂ | H-3, H-1αβ, H-2β | C-11, C-13, C-15, C-18, C-24-OAc | H-12β, H-22b |
| 3     | 0.55, m             | 17.9, CH₂ | H-22αβ, H-2αβ | C-1, C-2, C-4, C-5, C-10,  | H-22b                        |
| 4     | 16.1, C             | 42.1, C | H-6a/b       | C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-10, C-11-OAc, C-12, C-13 | H-23                         |
| 5     | 0.92, m             | 53.2, CH₂ | H-6aβ        | C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-10, C-11-OAc, C-12, C-13 | H-23                         |
| 6a    | 1.68, m             | 22.1, CH₂ | H-6bβ        | C-5, C-7, C-8, C-10         | H-6b                        |
| 6b    | 1.49, m             | 36.5, CH₂ | H-6aβ, H-7α  | C-5, C-7, C-8, C-10         | H-6a                        |
| 7β    | 2.42, m             | 36.5, CH₂ | H-6aβ, H-7α  | C-5, C-7, C-8, C-10         | H-6a                        |
| 7α    | 0.81, m             | 57.4, CH₂ | H-6αβ, H-7β  | C-6, C-8, C-9, C-14, C-24   | H-7β                         |
| 8     | 10.1, s             | 36.5, C | H-11         | C-6, C-8, C-9, C-14, C-24   | H-7β                         |
| 9     | 1.01, s             | 36.5, C | H-11         | C-6, C-8, C-9, C-14, C-24   | H-7β                         |
| 10    | 5.45, s             | 117.5, CH₂ | H-11         | C-6, C-8, C-9, C-14, C-24   | H-7β                         |
| 11    | 1.51, m             | 36.5, C | H-11         | C-6, C-8, C-9, C-14, C-24   | H-7β                         |
| 12β   | 2.07, m             | 57.4, CH₂ | H-11         | C-6, C-8, C-9, C-14, C-24   | H-7β                         |
| 12α   | 1.49, m             | 24.0, CH₂ | H-11         | C-6, C-8, C-9, C-14, C-24   | H-7β                         |
| 13    | 1.01, s             | 42.1, C | H-11         | C-6, C-8, C-9, C-14, C-24   | H-7β                         |
| 14    | 1.01, s             | 24.0, CH₂ | H-11         | C-6, C-8, C-9, C-14, C-24   | H-7β                         |
| 15a/b | 2.27, m             | 24.0, CH₂ | H-14         | H-16                         | H-16                         |
| 16    | 5.49, s             | 117.5, CH₂ | H-20αβ, H-18, H-15αβ | H-20b, H-15αβ                | H-20b, H-15αβ                |
| 17    | 5.04, s             | 135.6, C | H-16         | H-20b                        | H-20b                        |
| 18    | 2.15, m             | 62.7, CH₂ | H-16, H-19   | H-16, H-20b                  | H-20b                        |
| 19    | 5.24, d (4.4)       | 54.9, CH₂ | H-16         | H-16, H-20b                  | H-20b                        |
| 20a   | 4.44, d (12.2)      | 68.8, CH₂ | H-16         | H-16, H-20b                  | H-20b                        |
| 20b   | 4.15, d (12.2)      | 68.8, CH₂ | H-16         | H-16, H-20b                  | H-20b                        |
| 21    | 0.94, s             | 23.3, CH₃ | H-16         | H-16, H-20b                  | H-20b                        |
| 22a   | 0.43, d (3.9, 9.2)  | 22.7, CH₂ | H-3, H-22b   | H-3, H-22b                   | H-3, H-22b                   |
| 22b   | -0.06, d (4.8)      | 22.7, CH₂ | H-3, H-22b   | H-3, H-22b                   | H-3, H-22b                   |
| 23    | 0.95, s             | 14.0, CH₃ | H-3, H-22b   | H-3, H-22b                   | H-3, H-22b                   |
| 24a   | 4.91, d (12.9)      | 64.2, CH₂ | H-3, H-22a   | H-3, H-22b                   | H-3, H-22b                   |
| 24b   | 4.81, d (12.9)      | 64.2, CH₂ | H-3, H-22a   | H-3, H-22b                   | H-3, H-22b                   |
| 25    | 0.98, s             | 16.1, CH₃ | H-3, H-22a   | H-3, H-22b                   | H-3, H-22b                   |
| 11-OAc| 2.06, s             | 21.9, CH₃ | H-3, H-22a   | H-3, H-22b                   | H-3, H-22b                   |
| 24-OAc| 2.08, s             | 21.9, CH₃ | H-3, H-22a   | H-3, H-22b                   | H-3, H-22b                   |

$^1$H (600 MHz), $^{13}$C NMR (150 MHz), all δ in ppm relative to CDCl₃ = 7.26/7.70. $^b$Multiplicities determined by DEPT.
The $^1$H NMR spectrum showed unusual upfield resonances, diagnostic for a cyclopropyl ring $H_2-22$ (δ $-0.06$ brt, $J$ = 4.8 Hz, δ 0.43 dd, $J$ = 3.9, 9.2 Hz). Furthermore, this spectrum proved the presence of the olefinic proton $H-16$ (δ 5.24 d, $J$ = 4.4 Hz), further confirm the relationship between these protons. Hence, the coupling constant of $J$ δ 4.4 Hz between $H-19$ and $H-18$, further diagnostic for a cyclopropyl ring $H-18$. Moreover, the cross peak between $H-19$ (δ 0.98 s), and $H-25$ (δ 1.40 brt, δ = 8.5 Hz), and $H-21$ (δ 0.92 m), angular methines $H-5$ (δ 0.06 brt, δ = 4.8 Hz, $J$ = 3.9, 9.2 Hz), $H-3$ (δ 0.55 m), and $H-22a$ (δ 0.43 dd, $J$ = 3.9, 9.2 Hz), $H-14$ and $H-18$ (δ 0.55 m), based on HMBC cross peaks between the protons $H_2-22$ and the carbon atoms $C-2$ (δ 19.0), $C-5$ (δ 53.2) and $C-21$ (δ 23.3). The entire assignment of all NMR data is given in Table 1.

The relative configuration was determined from proton coupling constants and NOE data (Table 1, Figure 4). NOESY cross peaks between $H-3$ (δ 0.55 m), and $H-22a$ (δ 0.43 dd, $J$ = 3.9, 9.2 Hz), $H_2-21$ (δ 0.94 s), and $H_2-23$ (δ 0.95 s), as well as between $H_2-23$ and $H_2-24a$ (δ 4.91 d, $J$ = 12.9 Hz), $H_2-24b$ (δ 4.81 d, $J$ = 12.9 Hz) and $H_2-25$ (δ 0.98 s), and between $H_2-25$ and $H-19$ (δ 5.24 d, $J$ = 4.4 Hz), indicated that these protons share the same orientation on the molecular plane. The chemical shifts of the angular methyl groups $CH_3-23$ (δ 14.0) and $CH_3-25$ (δ 16.1) suggested that all ring junctions are trans [58-60]. This was supported by NOESY cross peaks between $H-22b$ (δ $-0.06$ brt, $J$ = 4.8 Hz) and $H_5$ (δ 0.92 m), angular methines $H-5$ and $H-9$ (δ 1.01 s), $H-9$ and $H-14$ (δ 1.40 brt, $J$ = 8.5 Hz), and between $H-14$ and $H-18$ (δ 2.15 m), from which a shared α-orientation can be inferred. Moreover, the cross peak between $H-19$ (δ 5.24 d, $J$ = 4.4 Hz) and $H_2-25$ (δ 0.98 s), and a coupling constant of $J$ = 4.4 Hz between $H-19$ and $H-18$, further confirm the trans relationship between these protons. Hence,
the structure and relative configuration of 12-deacetoxy-4-demethyl-11,24-diacetoxy-3,4-methylenedioxyxoscaralin was determined. It needs to be noted that the molecule was unstable over time, especially in ring E, and a variety of degradation products formed by, inter alia, hydrolysis of the hemiacetal and loss of the acetoxy groups.

The new scalarane was also detected in Doriprismatica stellata egg ribbons and Spongia cf. agaricina (Figure 3). It was isolated from both samples (egg ribbons: 1 mg, 0.1% wet weight; sponge: 0.7 mg, 0.02% wet weight) and the identity was validated by comparison of the MS and NMR spectra.

**Antibacterial activity**

All ethyl acetate extracts from Doriprismatica stellata nudibranchs, egg ribbons and Spongia cf. agaricina showed antibacterial activity against the Gram-positive Arthrobacter crystallopoites (DSM 2017) in a first screening approach. The pure compound 12-deacetoxy-4-demethyl-11,24-diacetoxy-3,4-methylenedioxyxoscaralin, isolated from all three extracts, was active against the Gram-positive Bacillus megaterium (DSM 32) (Supporting Information File 1).

**Discussion**

In this study, the new scalarane-type sesterterpene 12-deacetoxy-4-demethyl-11,24-diacetoxy-3,4-methylenedioxyxoscaralin was isolated from Doriprismatica stellata nudibranchs (Gastropoda, Mollusca), their egg ribbons, and the associated sponge Spongia cf. agaricina (Demospongiae, Porifera), collected from Bunaken National Park (BNP, North Sulawesi, Indonesia). Nudibranchs and their egg ribbons revealed higher concentrations of the scalarane in comparison to the sponge, likely due to a continuous accumulation of this compound.

In general, scalarane sesterterpenes are bioactive metabolites, mainly isolated from marine sources, such as Dictyoceratida sponges and the nudibranchs that feed on them [7,25,29,33,56]. So far, only six scalaranes containing cyclopropane rings, constructed of C-4, C-19 and C-20, have been identified [61,62]. The new 12-deacetoxy-4-demethyl-11,24-diacetoxy-3,4-methylenedioxyxoscaralin shared high similarities with 12,24-diacetoxy-3,4-methylenedioxyxoscaralin, as proven in this study. Sesterterpenes are a rare terpene class, accounting for less than 2% of all known terpenoids, with only a few reports on their biosynthesis [72-76]. However, their frequent occurrence in marine organisms is striking and sponges are considered as the prime source of these terpenoids [25]. Yet determining the origin and in vitro production of these metabolites is anything but trivial. Sponges are known to host complex symbiotic communities, with up to 30–60% as microbial biomass [13,77]. These highly species-specific communities are most probably vertically transmitted [78] and were shown to share and cover various core functions of sponge metabolism by functionally equivalent symbionts, analogous enzymes, or biosynthetic pathways [16,79,80]. Another Spongia species, S. officinalis, was shown to harbour bacteria with terpenoid cyclases/protein prenyltransferases responsible for a wide chemodiversity of terpenoid natural products [14,81]. Besides, the marine fungi Penicillium spp. and Aspergillus spp. are often associated with sponge hosts and were found to produce various terpenoids as well [15,82,83]. Hence, if sponges are not the origin of these metabolites, it is tempting to argue that the sesterterpene biosynthesis could be performed or mediated by their microbial symbionts. This further indicates a close association, interconnectedness, and probable co-evolution between microorganisms, sponges and nudibranchs [9]. D. stellata was not only found to sequester and accumulate 12-deacetoxy-4-demethyl-11,24-diacetoxy-3,4-methylenedioxyxoscaralin from Spongia cf. agaricina, but to pass it on to the egg ribbons as well. This, in addition to its bioactivity, might suggest a biological role, either as protection against predation, fouling, or in the reproductive cycle, as mentioned in previous studies on nudibranch egg ribbons [17,34-36]. The antibacterial activity of 12-deacetoxy-
4-demethyl-11,24-diacetoxy-3,4-methylenedioxoscalarin could point towards a potential protective role against bacterial biofilm formation. Unfortunately, the metabolite was unstable over time and it was not possible to conduct further assays. Future studies on scalarane sesquiterpenes could reveal their full potential and true biological and ecological functions in complex, co-evolved communities.

Experimental

General experimental procedures

Optical rotations were measured with a Jasco DIP 140 polarimeter. UV and IR spectra were obtained using Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. All NMR spectra were acquired in base-filtered CDCl$_3$ using Bruker Avance 300 DPX or Bruker Ascend 600 with prodigy cryoprobe spectrometers. Spectra were referenced to residual solvent signals with resonances at $\delta_{\text{H/C}}$ 7.26/77.00 ppm (CDCl$_3$). Mass spectra were recorded on a microOTOF-Q mass spectrometer (Bruker) with ESI-source coupled with an HPLC Dionex Ultimate 3000 (Thermo Scientific) using an Agilent Zorbax Eclipse Plus C$_{18}$ column (2.1 $\times$ 50 mm, 1.8 $\mu$m) at a temperature of 45°C. MS data were acquired over a range from 100–3000 m/z in positive mode. Auto MS/MS fragmentation was achieved with rising collision energy (35–50 keV over a gradient from 500–2000 m/z) with a frequency of 4 Hz for all ions over a threshold of 100. UHPLC started with 90% H$_2$O containing 0.1% acetic acid. The gradient began after 0.5 min to 100% acetonitrile (0.1% acetic acid) in 4 min. 2 $\mu$L of a 1 mg/mL sample solution was injected to a flow of 0.8 mL/min. HRAPCIMS were recorded on LTQ Orbitrap XL mass spectrometer. HPLC was carried out on a Waters Breeze HPLC system equipped with a 1525$\mu$ dual pump, a 2998 DAD detector, and a Rheodyne 7725i injection system and with a Waters Alliance HPLC system equipped with a Waters 2695 separation module and a Waters 996 PDA detector. A Macherey-Nagel Nucleodur C$_{18}$ Pyramid column (250 mm $\times$ 10 mm; 5 $\mu$m) and a Phenomenex Kinetex C$_{18}$ column (250 mm $\times$ 4.6 mm, 5 $\mu$m) were used for separation.

Biological material

Samples of Doriprismatica stellata sea slugs (Nudibranchia, Gastropoda, Mollusca), their egg ribbons and pieces of the sponge, on which they were found (1.2 g, 0.7 g, and 3.5 g wet weight, respectively) were collected via scuba diving in August 2016 during another field trip to Bunaken National Park (BNP, North Sulawesi, Indonesia, 1° 37' 51'' N, 124° 45' 05'' E) at the coral reef drop off. Four additional D. stellata sea slugs (2.5 g wet weight) were collected in October 2016 during another field trip to BNP. The nudibranchs and associated egg ribbons were identified as Doriprismatica stellata by H. Wägele and N. Undap at the Zoological Research Museum Alexander Koenig, Bonn, Germany [84,85]. The sponge displayed a foliose habit with brownish-violet pigmentation and was identified as Spongia cf. agaricina using methods as described by Ackers et al. in 2007 [86], see also Erpenbeck et al. from 2020 [87] (Supporting Information File 1). Specimens were stored in ethanol (96%) at −20 °C until further extraction and processing in the laboratories at the University of Bonn. A part of the collected sea slug and substrate materials will be finally stored at the Sam Ratulangi University, Manado, Indonesia, in the Reference Collection under the numbers SRU2015/01 and SRU2016/02. A fraction of the sponge material is stored in the Bavarian State Collection for Paleontology and Geology under collection number SNSB-BSPG.GW41291.

Extraction and isolation

Six Doriprismatica stellata nudibranchs (3.7 g wet weight), their egg ribbons (0.7 g wet weight) and pieces of the associated sponge (3.5 g wet weight) were separately frozen, crushed and ultrasonicated for a total of 3 minutes (30 s intervals) on ice, while submerged in a minimum of first acetone (Ac) and consecutively methanol (MeOH). The ethanolic storage solutions of D. stellata nudibranch, egg ribbon, and Spongia cf. agaricina samples were each combined with the respective Ac/MeOH extracts of the samples and dried under vacuum to give the crude extracts. After liquid–liquid separation of the three crude extracts (0.9 g, 0.3 g, and 0.2 g, respectively) between 50 mL water (H$_2$O) and three times 50 mL ethyl acetate (EtOAc), EtOAc solubles (223 mg, 35 mg, and 81 mg) were separated by RP-HPLC. A Phenomenex Kinetex C$_{18}$ column (250 mm $\times$ 4.6 mm, 5 $\mu$m), with a linear gradient elution from 70:30 (MeOH/H$_2$O) to 100% MeOH in 25 min, and a flow of 1.5 mL/min was used for separation. The isolated metabolite had a retention time around 13 minutes.

12-Deacetoxy-4-demethyl-11,24-diacetoxy-3,4-methylenedioxoscalarin

C$_{29}$H$_{42}$O$_6$, white amorphous solid (12.7 mg); [a]$_{D}^{20} +40.5$ (c 0.6, CHCl$_3$); IR (ATR) $v_{\text{max}}$: 3416, 2922, 2861, 1732, 1234 cm$^{-1}$; $^{1}$H and $^{13}$C NMR (Table 1); HRAPCIMS (m/z): [M + H]$^{+}$ calcd. for C$_{29}$H$_{43}$O$_6$, 487.3060; found, 487.3054.

Supporting Information

Supporting Information File 1
Spectroscopic data and other relevant information for 12-deacetoxy-4-demethyl-11,24-diacetoxy-3,4-methylenedioxoscalarin.

[https://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-16-132-S1.pdf]
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ORCID® IDs

Dirk Erpenbeck - https://orcid.org/0000-0001-9947-8079
Till F. Schäberle - https://orcid.org/0000-0001-9947-8079
Gabriele M. König - https://orcid.org/0000-0003-4916

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