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## **A mini-review on oxysporone**

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## **ABSTRACT**

Oxysporone, possessing a 4*H*-furo(2,3-*b*)pyran-2(3*H*)-one structure, is a fungal metabolite, first isolated from *Fusarium oxysporum*. Later, this compound was also reported from the fungal genera, *Diplodia*, *Pestalotia* and *Pestalotiopsis*. Oxysporone was patented as an antibiotic for the treatment of dysentery, and its phytotoxic property has been well-established. Interesting biological and chemical properties of oxysporone make this compound attractive for its potential biotechnological applications in agriculture, especially as a new agrochemical with a lower environmental impact. However, it requires further extensive bioactivity screening looking at beyond its established phytotoxicity and preliminary antifungal property. It has a simple chemical structure (C<sub>7</sub>H<sub>8</sub>O<sub>4</sub>), and the hydroxyl group at C-4 provides options for synthesising numerous analogues by simply incorporating various functionalities at C-4, whilst keeping the main skeleton intact. This mini-review provides a critical overview on the occurrence, production, synthesis, structure elucidation and bioactivities of oxysporone.

## **KEYWORDS**

Oxysporone

Antibiotic

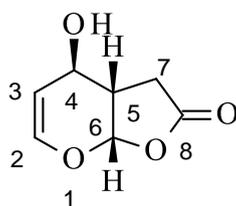
Endophytic fungi

*Fusarium oxysporum*

Structure elucidation

## 1. Introduction

Oxysporone (**1**, mol. wt. 156, mol. formula: C<sub>7</sub>H<sub>8</sub>O<sub>4</sub>), possessing a 4*H*-furo(2,3-*b*)pyran-2(3*H*)-one structure (Figure 1), is a fungal metabolite, first isolated from the Ascomycete fungal strain, *Fusarium oxysporum* (Figure 2), growing on earthworm cast in Nigeria, which is traditionally used along with salt to treat chronic dysentery (Adesogan and Alo, 1979). Later, this compound was patented as an antibiotic for the treatment of dysentery, and its phytotoxic property was established. Interesting biological and chemical properties of oxysporone (**1**) make this compound attractive for its potential biotechnological applications in agriculture, especially as a new agrochemical with a lower environmental impact (Andolfi et al., 2014). This mini-review aims to provide a critical account on the occurrence, production, synthesis, structure elucidation and bioactivities of oxysporone (**1**).



**Figure 1.** Structure of oxysporone (**1**)



**Figure 2.** *Fusarium oxysporum*

## 2. Distribution

Since the first isolation of oxysporone (**1**) from *Fusarium oxysporum*, it has been reported from a few other endophytic fungal species from other genera *Diplodia*, *Pestalotia* and *Pestalotiopsis* (Nagara and Ando, 1989; Venkatasubbaiah et al., 1991; Evidente et al.,

2012; Luo et al., 2012) (Table 1). An endophyte is a bacterium or fungus that lives within a plant for at least part of its life cycle without causing apparent disease. Endophytes are found in all plants studied to date, and whilst their specific functions are yet to be well-studied, it is assumed that some endophytes may enhance growth of the host plant, help in nutrient acquisition, increase tolerance of abiotic stresses, defense against insects, mammalian herbivores and plant pathogens. Some endophytic fungi have recently been shown to possess phytotoxicity causing irreversible damages to the host plant. Endophytic fungi exist widely inside the healthy tissues of living plants, and are important components of plant microecosystems.

Most of the fungal endophytes belong to the phylum Ascomycota. Vertically transmitted fungal endophytes are asexual and transmit via fungal hyphae penetrating the host's seeds. The genera *Pestalotia* and *Pestalotiopsis* are phylogenetically close and belong to the same family. The *Pestalotia* and *Pestalotiopsis* species of the Amphisphaeriaceae family are widely distributed all over the world; most of them are plant pathogens but many are saprobes in soil or on plant debris. *F. oxysporum* is a pervasive plant endophyte that can colonize plant roots and may even protect plants or be the basis of disease suppression.

**Table 1**

Sources of oxysporone (1)

| <b>Fungal strains</b>             | <b>Family</b>      | <b>References</b>             |
|-----------------------------------|--------------------|-------------------------------|
| <i>Diplodia africana</i>          | Botryosphaeriaceae | Evidente et al. (2012)        |
| <i>Fusarium oxysporum</i>         | Nectriaceae        | Adesogan and Alo (1979)       |
| <i>Pestalotia longiseta</i>       | Amphisphaeriaceae  | Nagata and Ando (1989)        |
| <i>Pestalotiopsis karstenii</i> * | Amphisphaeriaceae  | Luo et al. (2012)             |
| <i>Pestalotiopsis oenotherae</i>  | Amphisphaeriaceae  | Venkatasubbaiah et al. (1991) |

\* *Pestalotiopsis karstenii* does not produce oxysporone (1), but produces its derivatives

### **3. Production**

The production of any secondary metabolites from any endophytic fungus usually involves multiple steps, i.e., transfer of endophytic fungus from plant organs to culture medium, growth of the fungus, separation of individual strains (colonies), growing and identifying the strains based on morphological features and genetic analysis, growing the selected fungal strains in liquid culture, stopping the growth, filtration of the culture medium,

extraction with organic solvents (generally, ethyl acetate or  $\text{CHCl}_3$ ), chromatographic analysis to isolate individual secondary metabolites and finally, spectroscopic identification of the isolated compounds. It is worth mentioning that variation in the growing environment can have significant impacts on the quantity and diversity in the production of fungal metabolites (VanderMolen et al., 2013). Thus, optimization of growing conditions is essential prior to embarking on to any large-scale production of fungal metabolites from cultures.

All isolation protocols for oxysporone (**1**), published to date, also follow the above pathway. However, differences in culture media, media composition and extraction solvents could be observed in various methods. Oxysporone (**1**) was first produced from *Fusarium oxysporum*, which was cultured on the Czapek-Dox medium (Adesogan and Alo, 1979). The Czapek-Dox medium is composed mainly of sucrose, sodium nitrate, magnesium glycerophosphate, potassium chloride, dipotassium sulphate and ferrous sulphate. At the initial stage of the growth, there was no pigmentation, and the fungus yielded fusaric acid and its dehydro-analogue. However, the fungus pigmented heavily on Raulin-Thorn medium (Brian et al., 1946), which was mainly due to the production of non-volatile naphthazarins within the first four days. On the eighth day, a red oil was isolated from the culture medium, which contained oxysporone (**1**). Oxysporone (**1**) was finally purified by column chromatography on Silica gel using a mobile phase comprising ethyl acetate (EtOAc) and petrol (2:3) with a yield of about 15 mg/L of culture fluid.

Nagata and Ando (1989) produced oxysporone (**1**) from the Tea Gray Blight Fungus *Pestalotia longiseta* (synonym: *Pestalotiopsis longiseta*) which is a plant pathogen (Table 1). They still-cultured this fungal strain on a potato broth containing glucose (2%) and peptone (0.5%) at pH 6.2-6.3 and 28°C for five days under dark conditions. The filtered culture medium (800 mL) was extracted with EtOAc, and the extract was subjected to silica gel chromatography eluting with toluene-EtOAc (1:1) to obtain various fractions. The phytotoxic fraction was analysed by thin layer chromatography (TLC) on silica gel using a mobile phase of EtOAc-acetone (3:2) to obtain 166 mg of pure oxysporone (**1**). Later, this compound was also isolated from a culture of *Pestalotiopsis oenotherae*, which grows on evening primrose (*Oenothera laciniata*) (Venkatasubbaiah et al., 1991). Venkatasubbaiah et al. (1991) also used a similar potato-dextrose-based medium for culturing *P. oenotherae*, a well-known plant pathogen, using the shake flask culture method. The culture filtrate was extracted with EtOAc, and subsequently silica gel column chromatography using a solvent system comprising benzene-EtOAc mixture of increasing polarity, i.e., increasing amount of EtOAc, afforded 110 mg of

oxysporone (**1**) from the benzene-EtOAc (3:2) fraction after preparative-TLC purification on silica gel (mobile phase: 10% MeOH in CHCl<sub>3</sub>). The use of benzene as a chromatographic solvent, however, is no longer allowed in most laboratories because of its severe toxicity. Another species of the same genus, *Pestalotiopsis karstenii*, isolated from the stems of *Camellia sasanqua* collected from Nanning, Guangxi Province, China, was grown in a liquid fermentation culture to isolate four oxysporone (**1**) derivatives, including two new ones, but not oxysporone (**1**) itself.

Progressive dieback of shoots and branches of *Juniperus phoenicea*, a typical juniper, is commonly caused by the endophytic fungus, *Diplodia africana*, which is also known to cause wood necrosis on stone fruit trees in South Africa (Evidente et al., 2012). *D. africana* was also detected as the main pathogen involved in the aetiology of cankers and branch dieback of the *J. phoenicea* trees on Caprera Island, within the La Maddalena Archipelago in Sardinia, Italy (Andolfi et al., 2014). Evidente et al. (2012) isolated oxysporone (**1**), together with two other dihydrofuropran-2-one compounds from this fungal strain collected from symptomatic branch of *J. phoenicea* growing wild in Caprera Island (Italy). The fungus was identified based on morphological characters and analysis of Internal Transcribed Spacer (ITS) rDNA and EF1- $\alpha$  gene sequence. The culture method was quite similar to those described above, and was based on potato-dextrose-agar. Briefly, *D. africana* was grown in M1D medium (400 mL each, pH 5.7) in an Erlenmeyer flask (2 L each), seeded with a mycelia suspension (5 mL) and incubated at 25°C for four weeks in dark conditions. Multiple cultures were used. After incubation, the cultures were filtered and the filtrate (12 L) was acidified to pH 4 with 2M HCl and extracted with EtOAc (4 x 3 L). The combined EtOAc extracts were dried with Na<sub>2</sub>SO<sub>4</sub> and evaporated under reduced pressure to obtain a reddish brown oil (6 g), which was subjected to silica gel column chromatography and preparative-TLC to afford 356 mg of oxysporone (**1**) and other metabolites of varying amounts.

#### **4. Structure elucidation**

The structure of oxysporone (**1**) was first proposed by Adesogan and Alo (1979) on the basis of IR, <sup>1</sup>H (100 MHz) and <sup>13</sup>C NMR (25 MHz) (Table 2) and MS data analyses as well as by formation of its mono-acetate. The relative stereochemistry of the chiral centres was established relying on <sup>1</sup>H-<sup>1</sup>H coupling constant analyses and spectroscopic arguments. However, they could not assign individual <sup>1</sup>H NMR signals originating from the protons associated with C-5 and C-7, and also, the <sup>13</sup>C NMR assignment of C-5 and C-7 was incorrect,

which should be swapped. It is likely the NMR spectra were obtained in CDCl<sub>3</sub>, but it was not clearly mentioned. The EIMS spectrum confirmed the molecular formula by giving the molecular ion peak [M]<sup>+</sup> at *m/z* 156.042, which was consistent with the molecular formula C<sub>7</sub>H<sub>8</sub>O<sub>4</sub>. Ten years later, Nagata and Ando (1989) reported better-assigned <sup>1</sup>H (270 MHz) and <sup>13</sup>C NMR (67.8 MHz) data, where they corrected the assignment of <sup>13</sup>C NMR data for C-5 and C-7, and also assigned the <sup>1</sup>H NMR signals for the methine (H-5, δ2.90, m) and the methylene (H<sub>2</sub>-7, δ2.30, dd, *J* = 17.1 and 12.2 Hz; 2.59, dd, *J* = 17.1 and 8.1 Hz) protons, They also assigned the -OH proton signal at δ1.96 as a broad doublet. In addition to IR, EIMS and NMR, HRMS and optical rotation data were obtained; at 25°C, the [α]<sub>D</sub> was +39.5 (c = 0.9%, MeOH).

**Table 2**

<sup>1</sup>H and <sup>13</sup>C NMR data of oxysporone (**1**) (Adesogan and Alo, 1979)

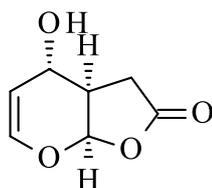
| Position | Chemical shift (δ) in ppm  |                     |
|----------|--|---------------------|
|          | <sup>1</sup> H NMR (Coupling constant <i>J</i> in Hz in parentheses) | <sup>13</sup> C NMR |
| 2        | 6.37, 1H, d (6.0)  | 143.5               |
| 3        | 5.06, 1H, ddd (6.0, 5.5, 1.0)  | 100.1               |
| 4        | 4.16, 1H, dd (5.5, 2.0)  | 60.0                |
| 5        | 2.00-3.00*   | 29.5 <sup>a</sup>   |
| 6        | 5.82 1H, d (4.5)   | 96.0                |
| 7        | 2.00-3.00*   | 41.8 <sup>a</sup>   |
| 8        | -  | 175.3               |
| 4-OH     | 2.00-3.00*   | -                   |

\*Overlapped peaks integrated for 4H, but could not be individually assigned; <sup>a</sup>Incorrect assignment, and should be swapped.

Venkatasubbaiah et al. (1991) isolated oxysporone (**1**) from *Pestalotiopsis oenotherae* and obtained <sup>1</sup>H (300 MHz) and <sup>13</sup>C NMR (75 MHz) data, which were almost identical to those reported previously. However, to the best of our knowledge, no 2D NMR data are available to date. Venkatasubbaiah et al. (1991) confirmed that this compound does not absorb UV light above 220 nm and the λ<sub>max</sub> was 211 nm to be exact. They also reported the CIMS data. The EIMS data of this compounds were reported as follows: *m/z* (rel. int.) 156 [M]<sup>+</sup> (10), 138 (10), 128 (10), 114 (30), 97 (20), 84 (100), 73 (60), 71 (50), 55 (80) and 43 (30), and the IR

absorptions are at  $\nu^{\text{film}} \text{ cm}^{-1}$  : 3350 (br), 1785 (s) ( $\gamma$ -lactone) and 1645 (s) (CH=CH-O-) (Venkatasubbaiah et al., 1991).

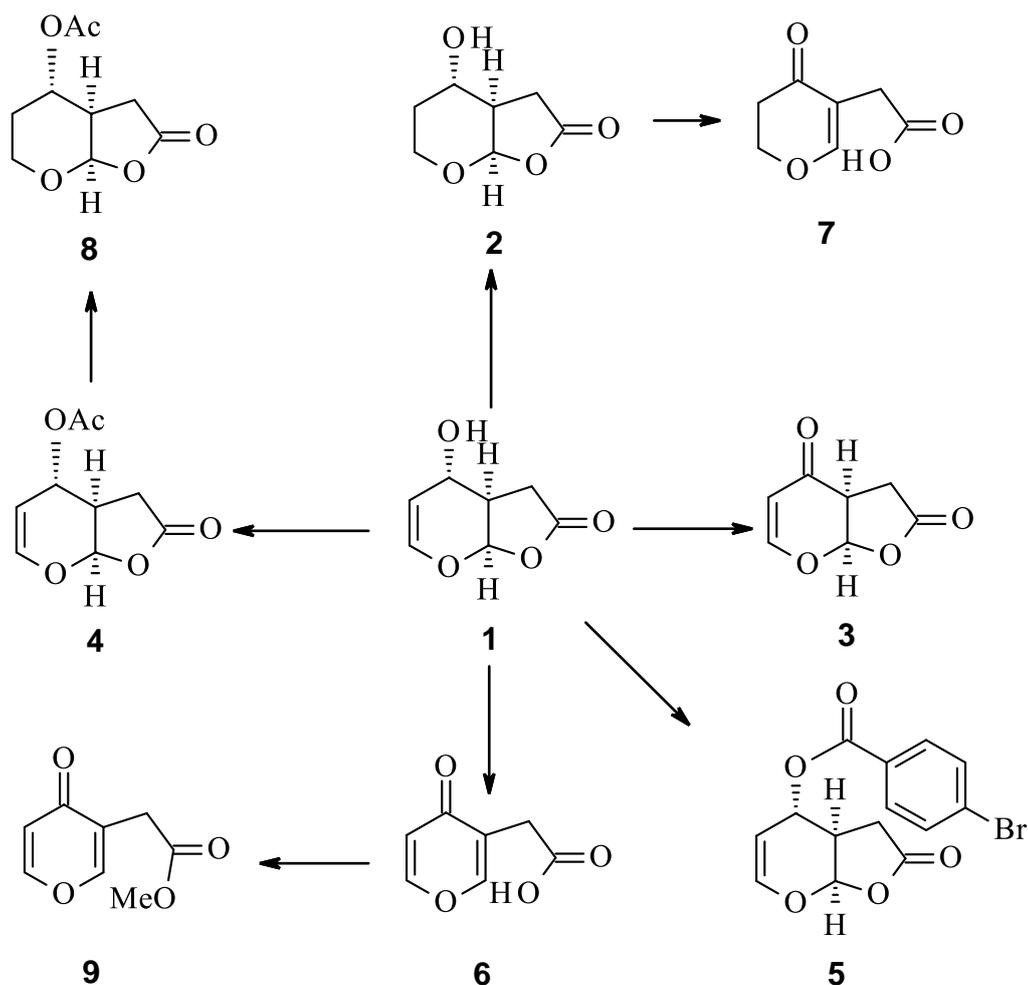
The absolute configuration of oxysporone (**1**) (Figure 3) was not known until 2013. Mazzeo et al. (2013) first established the absolute configuration (4*S*,5*R*,6*R*) of this compound by chiroptical methods, notably, optical rotatory circular dichroism (ORD), electronic circular dichroism (ECD) and vibrational circular dichroism (VCD) studies along with the extensive uses of computational techniques and mathematical modelling. Considering the known (4*R*\*,5*S*\*,6*S*\*) relative configuration of oxysporone (**1**) (Figure 1), conformational analysis was performed on the (4*R*,5*S*,6*S*) enantiomer, offering six conformers (within a 10 kcal/mol energy window), which differed by the orientation of the 4-OH group and the sense of twist of the five- and six-membered rings (Mazzeo et al., 2013). For this analysis, the Spartan 02 package incorporating the MMFF94s molecular mechanics force field and Monte Carlo search on chosen absolute configurations was used. It was suggested that the hydroxy group occupies both *pseudo*-equatorial and *pseudo*-axial orientations. All conformers were fully optimized using the density functional theory (DFT) analyses (DFT/B3LYP level of theory). After analysing the conformational space, the geometries within a 10 kcal/mol energy window were subjected to *ab initio* energy minimization as implemented in the Gaussian 09 package. The structure of oxysporone (**1**) was subjected to the DFT/B3LYP level of theory by resorting to four combinations of basis sets as well as an implicit solvation model: gas-phase/6-31G(d), gas-phase/cc-pVDZ, gas-phase/6-311++G(2d,2p), gas-phase/aug-cc-pVTZ, implicit solvation IEFPCM-6-31G(d) (Mazzeo et al., 2013).



**Figure 3.** Absolute configuration (4*S*,5*R*,6*R*) of oxysporone (**1**)

## 5. Synthesis

There has been no report on the total synthesis of oxysporone (**1**) available in the literature. However, the synthesis of several analogues (**2-9**) of this compound was reported (Andolfi et al., 2014); eight derivatives of **1**, four of which being novel compounds, were prepared by chemical transformation of **1** (Figure 4).



**Figure 4.** Oxysporone derivatives (2-9)

A simple acetylation of **1** by pyridine and acetic anhydride at room temperature afforded 4-*O*-acetyloxysporone (**4**). A hydrogenation reaction on **1** catalysed by Pt (5%) in MeOH at room temperature and atmospheric pressure under stirring gave 2,3-dihydrooxysporone (**2**). To an oxysporone (**1**) solution in dichloromethane, the Corey's reagent was added, and the reaction mixture was stirred at room temperature for 1 h to synthesise 4-*O*-dehydrooxysporone (**3**). A solution of **1** in acetonitrile was treated with dimethylaminopyridine (DMAP) and *p*-bromobenzoyl chloride, stirred at room temperature for 4 h to obtain 4-*O*-*p*-bromobenzoyloxysporone (**5**). Jones' reagent was added, under constant stirring, to a solution of oxysporone (**1**) in acetone at 0°C to produce 2-(4-pyronyl)acetic acid (**6**). 4-*O*-Acetyl-2,3-dihydrooxysporone (**8**) was synthesised from compound **4** by hydrogenation involving a presaturated suspension of Pt (5%) in MeOH carried out at room temperature for 5 h. Compound **2** was converted to 2-(2,3-dihydro-4-pyronyl)acetic acid (**7**) using the Jones'

reagent. To a methanolic solution of compound **6**, an ethereal solution of  $\text{CH}_2\text{N}_2$  was added until a yellow colour was persistent yielding methyl ester of 2-(4-pyronyl)acetic acid (**9**).

## 6. Bioactivity

Bioactivity studies on oxysporone (**1**), surprisingly, have been limited to its phytotoxicity and antifungal property over the last few decades. Oxysporone (**1**) was first isolated as a potential cure for chronic dysentery (Adesogan and Alo, 1979), and later, its phytotoxic property was identified (Nagata and Ando, 1989; Venkatasubbaiah et al., 1991; Hirota et al., 1993; Evidente et al., 2012).

Evidente et al. (2012) evaluated the phytotoxic property of oxysporone (**1**) and related compounds using the leaf puncture assay and the cutting assay. In the leaf puncture assay, tomato (*Lycopersicon esculentum* var. *Marmande*), holm oak (*Quercus ilex*) and cork oak (*Q. suber*) leaves were used. All test compounds including oxysporone (**1**) were dissolved in MeOH and diluted with distilled water to obtain the final concentration (4% MeOH), and from there, 1.0, 0.5, 0.25 and 0.1 mg/mL concentrations were used. The test solutions (20  $\mu\text{L}$ ) were applied on the axial side of leaves that had previously been needle punctured, and droplets (20  $\mu\text{L}$ ) of MeOH in distilled water (4%) were applied on leaves as control. The leaves were kept in a moist chamber to prevent drying, observed daily and scored for symptoms after one week. The effect of the phytotoxins on the leaves, consisting in necrotic spots surrounding the puncture, were observed up to three weeks. Lesions were estimated using APS Assess 2.0 software (<http://aps-assess.software.informer.com/2.0b/>) following the tutorials in the user's manual. The lesion size was expressed in  $\text{mm}^2$ . Phytotoxicity was also tested on apical parts of the excised twigs (10 cm long) of *Juniperus phoenicea*. The cuttings were taken from 3-year-old seedlings of this plant grown in the greenhouse at 25°C. Compounds were tested at 0.2, 0.1 and 0.05 mg/mL. The toxicity of these solutions was assessed by placing the test plant parts in the assay solution (2 mL) and then transferring them to distilled water. Symptoms were visually evaluated up to 30 days. The phytotoxicity of all compounds was again tested on tomato cuttings taken from 21-day-old seedlings, and the symptoms were visually evaluated up to one week.

Oxysporone (**1**) was found to be the most phytotoxic among the test compounds. In the leaf puncture assay, oxysporone (**1**) exhibited considerable phytotoxicity at a range of concentrations from 0.1 to 1 mg/mL, causing necrotic lesions (necrosis area ranging from 2 to

30 mm<sup>2</sup>) to leaves of all species tested. In the tomato cuttings assay, the cuttings treated with 0.2 and 0.1 mg/mL of **1** displayed complete wilting within 48 h from the application. Symptoms of phytotoxicity (stewing on stem) were also noticed with this compound at the lowest concentration tested (0.05 mg/mL). In the assay using the excised twigs of *Juniperus phoenicea*, oxysporone (**1**) caused yellowing and browning on leaves and twig dieback at 0.2 and 0.1 mg/mL. Symptoms induced by oxysporone (**1**) were similar to those observed in the field.

Andolfi et al. (2014) carried out a detailed bioactivity studies, e.g., phytotoxicity and antifungal property, on oxysporone (**1**) and its derivatives. Leaf puncture assay, tomato cutting assay and antifungal assay were performed. Oxysporone (**1**) and its derivatives (**2-9**) (Figure 4) were tested on non-host plants and on four destructive plant pathogens including two fungal species, *Athelia rolfsii* and *Diplodia corticola*, and two Oomycetes, *Phytophthora cinnamomi* and *P. plurivora*. As previously reported, oxysporone (**1**) came out as the most phytotoxic of all test compounds. From the analysis of the structures and the activities displayed by oxysporone (**1**) and its derivatives, structure–activity relationships (SAR) and some key structural features related to the phytotoxic activity could be established. It appeared that the dihydrofuropyran carbon skeleton is a structural feature indispensable for the activity, the double bond of the dihydropyran ring present in **1** is also an important structural feature for phytotoxic activity, and the hydroxy group at C-4 is somehow associated with the phytotoxic effects.

## 6. Concluding remarks

Oxysporone (**1**) is an interesting antibiotic, which requires further extensive bioactivity screening looking at beyond its established phytotoxicity and preliminary antifungal property. It has a simple structure and the hydroxyl group at C-4 provides ample opportunities to synthesise numerous analogues by simply incorporating various functionalities at C-4 whilst keeping the main skeleton intact.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Acknowledgement

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