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# OCCURRENCE OF CHOLECALCIFEROL IN THE CALCINOGENIC PLANT TRISETUM FLAVESCENS

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Summary

The grass Trisetum flavescens causes severe calcification of soft tissues upon ingestion by various species, which has been ascribed by others to a $1,25(OH)_2$ vitamin $D_3$-like activity.

By a special purification procedure involving high pressure liquid chromatography and continuous biological testing the active principle was purified. By means of GC/MS it was identified as cholecalciferol, being present in a concentration of about 0.1 ppm in the lyophylized plant dry matter. $1,25(OH)_2$ vitamin $D_3$ or other metabolites of vitamin $D_3$ were not present. Since such low concentrations could hardly explain the calcinosis observed, a more active "bound form" of vitamin $D_3$ may be present in Trisetum flavescens.

Certain plants in different parts of the world are known to cause calcinosis in cattle, sheep, horses and other animals. The classical example of plant poisoning resulting in chronic calcification of soft tissues is called Espichamento in Brazil and Enteque seco in Argentine[(1)]. The disease which shows great similarity to vitamin D-intoxication is due to the ingestion of the plant Solanum malacoxylon.

A disease similar to Solanum malacoxylon poisoning was described in horses and cattle in Florida, associated with the intake of Cestrum diurnum, which is a Solanacee too[(2)].

In the Alpine region of Germany and Austria a disease called "enzootic calcinosis" has been described[(3)(4)]. The clinical symptoms are the same as with the other calcinogenic plants: loss of weight, bent forelegs, reduced milk production, emaciation, increased heart rate and possibly death.

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Autopsy reveals extensive calcification in the soft tissue, the cardio-vascular system and in lungs and kidneys.
Since neither Solanum malacoxylon nor Cestrum diurnum occurs in these regions, systematical investigations on the etiology of the calcinosis were started. Finally it has been clearly shown by Dirksen[5] that the enzootic calcinosis is produced by the ingestion of the grass Trisetum flavescens.

From Solanum malacoxylon a water soluble sterol-glycoside has been isolated, which contains $1,25(OH)_2$ vitamin $D_3$, the active metabolite of vitamin $D_3$[6]. The calcinogenic principle of Cestrum diurnum has also been reported to contain $1,25(OH)_2$ vitamin $D_3$-like activity[7], though it may contain the hormone linked to fewer carbohydrate moieties. These results were obtained by physiological studies as well as by gas chromatographic, chemical and mass spectrographic analysis[8].

The similarity of the symptoms of Trisetum flavescens to those of other calcinogenic plants has initiated the search for $1,25(OH)_2$ vitamin $D_3$ too. - So Wasserman et al.[9] were able to induce the synthesis of the vitamin D dependent calcium binding protein by feeding Trisetum flavescens to rachitic chicks, but the grass did not overcome the strontium inhibition of the kidney 25-hydoxycholecalciferol-1-hydroxylase. From these results they suggested that there is no $1,25(OH)_2$ vitamin $D_3$-like activity and the mechanism by which Trisetum flavescens causes calcinosis differs from that of Solanum malacoxylon[9].

In contrast to Wasserman[9], Peterlik et al. obtained evidence for a $1,25(OH)_2$ vitamin $D_3$-like activity though both authors were using the same procedure of inhibiting the renal $1\alpha$-hydroxylase system by high concentrations of strontium[10]. Zucker et al. who isolated the calcinogenic principle of Trisetum flavescens by high pressure liquid chromatography suggested a vitamin D-like activity[11], and Rambeck et al.[12] demonstrated the non-identity of the active principle of Trisetum flavescens with $1,25(OH)_2$ vitamin $D_3$.

Therefore, the present investigations were undertaken to study the active principle by analytical methods combined with a bioassay to reveal if vitamin D or one of its metabolites occur in Trisetum flavescens.

## Materials and Methods

Extraction and purification of the active principle. Trisetum flavescens, harvested in August 1977, was lyophylized, extracted with diethyl ether, and saponified with potassium hydroxide in ethanol. The nonsaponifiable material was chromatographed on a 20 x 2 cm column packed with $Al_2O_3$. The elution was performed with increasing amounts of diethyl ether in light petroleum. All fractions were monitored by thin layer chromatography and tested for biological activity by measuring the serum phosphate of rats according to Zucker[11]. The fraction eluted with 9 % diethyl ether in light petroleum, which was the only one that contained the active principle, was further purified on a preparative scale by high pressure liquid chromatography. A Waters liquid chromatograph was employed for this work. The biological fraction was then further separated on a high efficiency silica column (μ-Porasil) followed by an analysis on a reversed phase system (μ-Bondapak). Solvent systems were 2.5 % isopropanol in hexane for μ-Porasil and acetonitrile for μ-Bondapak. The UV-absorption of the effluent was recorded continuously at 254 nm and fractions corresponding to the chromatographic peaks were collected and tested for biological activity.

For peak identification a standard solution containing cholecalciferol, 1∝(OH) vitamin $D_3$, 25(OH) vitamin $D_3$ and 1,25$(OH)_2$ vitamin $D_3$ was added to an aliquot of the purified active fraction and the mixture again chromatographed on the reversed phase system.

### GC/MS Analysis

The residue obtained by evaporation of the biologically active HPLC fraction was dissolved in 40 microliters of silylating agent (pyridine/bis-(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane: 1/1/0.01). Quantitative silylation is achieved after 10 to 15 minutes at room temperature. One microliter of this reaction mixture was injected into a GC/MS system. This quantity corresponds to the extract of 2.3 - 3.4 g of Trisetum flavescens.

The gas chromatographic separation was carried out on a glass column of 2 m length, 3.2 i.d., packed with 5 % OV-17 (or 2 % SE-30) on gaschrom Q 80-100 mesh. The analysis was carried out with a linear temperature program: Initial temperature was 200 °C; a programing rate of 3 °C/min. or 6 °C/min. up to a limit of 300 °C was used. The carrier gas was helium with a flow rate of 30 ml/min.

The gas chromatograph model 1740 (Varian-Aerograph) was connected to a mass spectrometer CH 7 (Varian) by means of a two stage glass fritt separator (Biemann-Watson type). During preliminary studies some mass spectra were obtained by a Finnigan instrument[+].

## Results and Discussion

A high pressure liquid chromatographic analysis of the purified extracts of Trisetum flavescens together with vitamin $D_3$, 1∝(OH) vitamin $D_3$, 25(OH) vitamin $D_3$ and 1,25$(OH)_2$ vitamin $D_3$ (Fig. 1) demonstrates that the active principle has a retention time identical to

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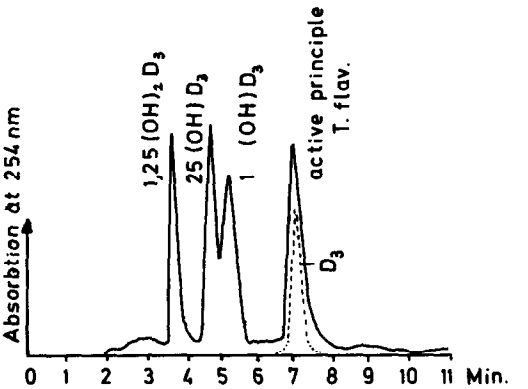


Figure 1: High speed liquid chromatography of a mixture of 1,25(OH)$_2$ vitamin D$_3$, 25(OH) vitamin D$_3$, 1 α (OH) vitamin D$_3$, vitamin D$_3$ and a purified extract of Trisetum flavescens on a reverse phase column (μ-C18). The compounds were eluted with acetonitrile, flowrate was 1.5 ml/min.

that of cholecalciferol. 1,25(OH)$_2$ vitamin D$_3$ has an elution time which is about half of that of the calcinogenic principle. It is clear from these results that the calcinogenic principle is not identical with a hydroxylated form of vitamin D$_3$.

Fig. 2 represents a typical gas chromatogram of the silylated biologically active fraction obtained with a SE-30 column.
The mass spectrum of the peak (10) is identical to the mass spectrum of silylated pyrocholecalciferol.

This latter compound is the main pyrolysis product of cholecalciferol in the GC[13]. This assignment of peak (10) is further confirmed by the gas chromatographic retention time on both stationary phases which were used. Isopyrocholecalciferol which is another pyrolysis product of cholecalciferol in the GC[13] was recognized as an isolated peak (13a) when using the SE-30 column, whereas with OV-17 as a stationary phase this compound could only be detected mass spectrometrically on the back side of the cholesterol peak (13).

The presence of pyro- and isopyrocholecalciferol in the residue of the active HPLC fraction proves that Trisetum flavescens contains vitamin D$_3$.

The main mass fragments in the mass spectra of silylated pyro- and isopyrocholecalciferol are listed in Table 1.

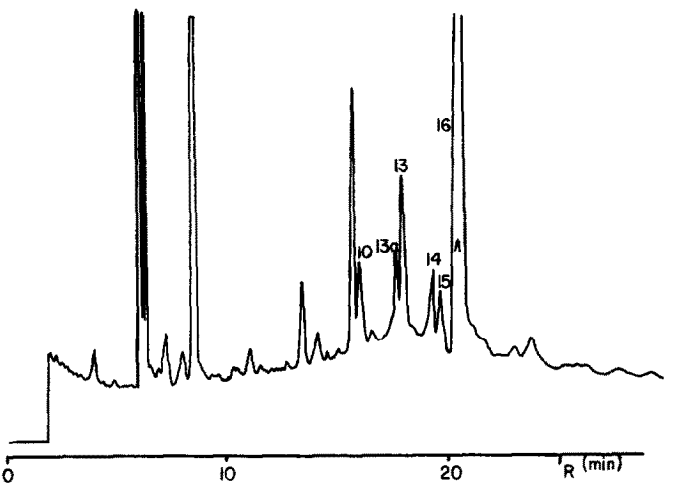


Figure 2: Gas chromatogram of the silylated biologically active fraction obtained with a SE-30 column.

Table 1

Abundance of the characteristical fragment ions in the mass spectra of pyro- and isopyrocholecalciferol as trimethylsilyl derivatives.

| m/e | A % pyro-cholecalciferol | A % isopyro-cholecalciferol |
|---|---|---|
| 456 $M^+$ | 15 | 37 |
| 441 | 3 | 8 |
| 366 | 15 | 47 |
| 351 | 100 | 100 |
| 325 | 88 | 77 |
| 253 | 13 | 54 |
| 211 | 27 | 57 |

Further sterols which could be detected in the active HPLC fraction are cholesterol (13), campesterol (14), stigmasterol (15) and ß-sitosterol (16).

Since neither $1,25(OH)_2$ vitamin $D_3$ nor another known metabolite of cholecalciferol were present, cholecalciferol itself must account

for at least the major part of the "vitamin D-like activity" in Trisetum flavescens. Based on estimates derived from GC and HPLC-measurements, a concentration of approximately 1 µg of cholecalciferol per 10 g of dry matter is present in Trisetum flavescens.

This amount seems by far not sufficient to explain the calcinogenic effect of Trisetum flavescens, since concentrations of vitamin $D_3$ considerably higher than 4000 I.U./kg of dry matter have no such effects.

Presumably, vitamin $D_3$ occurs in Trisetum flavescens in a bound form. This bondage may be broken during the saponification procedure. The postulated bound form might act differently and so cause calcinosis at a concentration at which free vitamin $D_3$ is nontoxic.

Further investigations are in progress.

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