A Vitamin D_3 Steroid Hormone in the Calcinogenic Grass *Trisetum flavescens*

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The grass *Trisetum flavescens* (golden oat grass, Goldhafer) causes soft tissue calcification in cattle and in sheep. The calcinogenic principle of the plant is the active vitamin D steroid hormone 1,25-Dihydroxyvitamin D_3, the major physiological regulator of calcium homeostasis in higher animals. From comparison with synthetic vitamin D metabolites in different bioassays, it is concluded that *T. flavescens* contains the 25-glucoside of $1,25(OH)_2D_3$. This compound, or rather the $1,25(OH)_2D_3$ liberated by ruminal fluid, is the calcinogenic factor of the grass.

Introduction

Grazing cattle and other herbivores in the Alpine region of Germany, Austria and Switzerland develop a disease called enzootic calcinosis [1–3]. The symptoms and signs in the affected animals are extensive soft tissue calcification, especially of the cardiovascular system, kidney, lungs, tendons and ligaments. The animals lose weight, their forelimbs become stiff, their backs are arching and the milk yield is reduced [4]. The disease can be of considerable economic importance. A similar calcinosis of grazing animals occurring in Argentina and in Brazil is caused by the ingestion of *Solanum malacoxylon* [5]. However, since this Solanaceae is unknown in Europe, the aetiology must be different.

Trisetum flavescens (golden oat grass, Goldhafer, avoine jaunatre) a common Graminae growing on Alpine pastures above 500 m, was identified by Dirksen *et al.* [6, 7] to be the cause of the enzootic calcinosis in the Alpine region. The calcinogenic activity is not restricted to grazing animals but can also be induced experimentally in rabbits [8]. Physiological studies showed that the toxic plant causes an enhanced absorption of phosphorus and calcium, resulting in a considerable hyperphosphatemia and in serum calcium levels in the upper part of the physiological range [9]. Most of these symptoms are known from hypervitaminosis D and it was therefore not surprising when a strong antirachitic activity of the plant was demonstrated [10–12].

From a diethyl ether extract of *Trisetum flavescens* we isolated a fraction showing a strong vitamin D-like activity in several bioassays. A gas chromatographic–mass spectrometric analysis of the purified extract revealed that the calcinogenic plant actually contains vitamin D_3, the "animal vitamin D" [13], at a concentration of about 0.1 ppm [14]. In analogy to the formation of the vitamin in the skin of vertebrates, vitamin D_3 in *T. flavescens* is synthesized only under the influence of UV light [15]. Part of the vitamin D_3 is present as an ester, but neither the vitamin itself nor the esterified form would be calcinogenic at this concentration [16, 17].

Peterlik *et al.* [18] were the first to report a vitamin D-like substance in *Trisetum flavescens* that requires no further metabolism in order to exert its effects. Active compounds are $1,25(OH)_2D_3$, $1,25(OH)_2D_2$, $1\alpha(OH)D_3$ or structural analogs. Furthermore, evidence for an aqueous soluble vitamin D-like substance in the calcinogenic grass was provided [19, 20]. These findings and our results from everted gut sac transport studies in rats indicated the presence of a substance able to mimick the action of $1,25(OH)_2D_3$ [21]. The aim of this study was to find out which 1α-hydroxylated vitamin D metabolite occurs in *Trisetum flavescens* and whether it is a glycosidic-binding form that renders the compound water soluble.

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Material and Methods

T. flavescens was grown at the "Lehr- und Versuchsgut Schleißheim der Universität München". Vitamin D metabolites and their glycosides were kindly supplied by Dr. W. Meier, Pharmaceutical Dept., F. Hoffmann-La Roche & Co. Ltd., Basle.

In the prophylactic chick assay, newly hatched male broiler chicks were put on a vitamin D-deficient diet containing added levels of vitamin D_3 or its derivatives. After three weeks weight gain, serum calcium and per cent bone ash of phalanges I and II of the middle toes were determined.

In the curative assay the chicks were fed a vitamin D-deficient diet for ten days. During the following experimental period vitamin D_3 or its derivatives were added to the diet. After six days the animals were killed and serum calcium and duodenal calcium-binding protein (CaBP) were determined.

For the curative Japanese quail assay newly hatched animals were put on a vitamin D-deficient diet for ten days. After this period, a diet containing vitamin D or vitamin D derivatives was fed for five consecutive days. Weight gain, serum calcium and bone ash per cent were determined.

The curative X-ray test in rats was performed as described by Weiser [22]. The degree of healing was assessed by scoring values. Compounds were given per stomach tube in propylene glycol/ethanol 10:1.

CaBP was determined by the ion exchange procedure according to Wasserman *et al.* [23]. CaBP is expressed as the percentage of radioactivity in the supernatant from total radioactivity per mg of protein.

Determination of $1,25(OH)_2D$ in serum and the assessment of the binding capacity of *T. flavescens* extract to the intestinal $1,25(OH)_2D_3$ receptor was carried out by the competitive protein-binding assay according to Mallon *et al.* [24].

Results and Discussion

While vitamin D_3 in *T. flavescens* was found in the ether extract of the plant, the vitamin D metabolite-like compound occurred in the residue of the ether extraction or in the aqueous extract. When this fraction of the calcinogenic grass was fed to rats, $1,25(OH)_2D$ concentration in serum increased (Table I). Similar results were obtained when *T. flavescens* was fed to cows [25].

Table I. $1,25(OH)_2D$ concentration in rat serum after feeding *T. flavescens* (residue of diethyl ether extraction).

Addition per kg of diet	$1,25(OH)_2D$ pg/ml serum (pool from 6 animals)
– (neg. control)	57
90 g *T. flavescens*	106
200 g *T. flavescens*	375

The increase in rat serum $1,25(OH)_2D$ concentration was not necessarily due to $1,25(OH)_2D_3$, since the competitive protein-binding assay used responds to $1,25(OH)_2D_3$ as well as to $1,25(OH)_2D_2$. The same is true for our finding that a water extract of *T. flavescens* was able to replace radioactive $1,25(OH)_2D_3$ from its intestinal receptor and therefore contained $1,25(OH)_2D$.

In order to find out which vitamin D metabolite is present in this plant, we compared the bioactivity of $1,25(OH)_2D_3$, $1,25(OH)_2D_2$ and *T. flavescens* in different bioassays. While in rats the two vitamin D metabolites and the extract of the grass behaved very similarly [26, 27], there was a fundamental difference in rachitic chicken. The synthesis of CaBP, which is a good indicator of the molecular expression of the hormonal action of vitamin D metabolites, is shown in Table II.

From these and other results [27] it can be concluded that $1,25(OH)_2D_2$ is about 10 times less active in chicken than $1,25(OH)_2D_3$, while the calcinogenic compound in *T. flavescens* shows a similar antirachitic activity in rats and in chickens. Thus all biological evidence indicates that the active principle is $1,25(OH)_2D_3$ and not $1,25(OH)_2D_2$.

The apparent paradox that the vitamin D metabolite in *T. flavescens* is water soluble can be explained

Table II. Synthesis of duodenal CaBP in rachitic chicks. Curative assay. Mean values ± S.D. (n = 10 per group).

Addition in nmol kg of diet	CaBP
– (neg. control)	1.24 ± 0.33
1.3 $1,25(OH)_2D_3$	2.46 ± 0.63
2.6 $1,25(OH)_2D_3$	4.17 ± 1.24
5.2 $1,25(OH)_2D_3$	5.06 ± 1.25
10.4 $1,25(OH)_2D_2$	3.09 ± 1.14
20.8 $1,25(OH)_2D_2$	4.01 ± 1.13
41.6 $1,25(OH)_2D_2$	6.09 ± 1.89
200 g *T. flavescens* ether extract residue	4.28 ± 1.18

by the finding that $1,25(OH)_2D_3$ in *T. flavescens* is present as a glucoside. Treatment with glucosidases or with ruminal fluid releases the free steroid hormone [28]. In *Solanum malacoxylon*, the calcinogenic plant of South America, also a glycoside of $1,25(OH)_2D_3$ is the active principle, but neither the nature of the carbohydrate moiety nor the connection to the aglycone have been elucidated.

Recently, Fürst *et al.* [29] synthesized for the first time β-D-glucopyranosides of some hydroxylated vitamin D metabolites. We compared the bioactivity of *T. flavescens* with two $1\alpha(OH)D_3$ glucosides and with the three possible $1,25(OH)_2D_3$ glucosides. In Fig. 1, the calcium excretion *via* the egg shell of Japanese quails is shown. At the concentration administered, neither $1\alpha(OH)D_3$-3-glucoside nor the disaccharide $1\alpha(OH)D_3$-3-cellobioside increased Ca excretion *via* the egg shell. In a curative X-ray test in rats, where the degree of calcification of the epiphyseal fuge of the tibia is measured, the bioactivity of the $1\alpha(OH)D_3$ glucosides was also low [30]. Of the three possible glucosides of $1,25(OH)_2D_3$ the

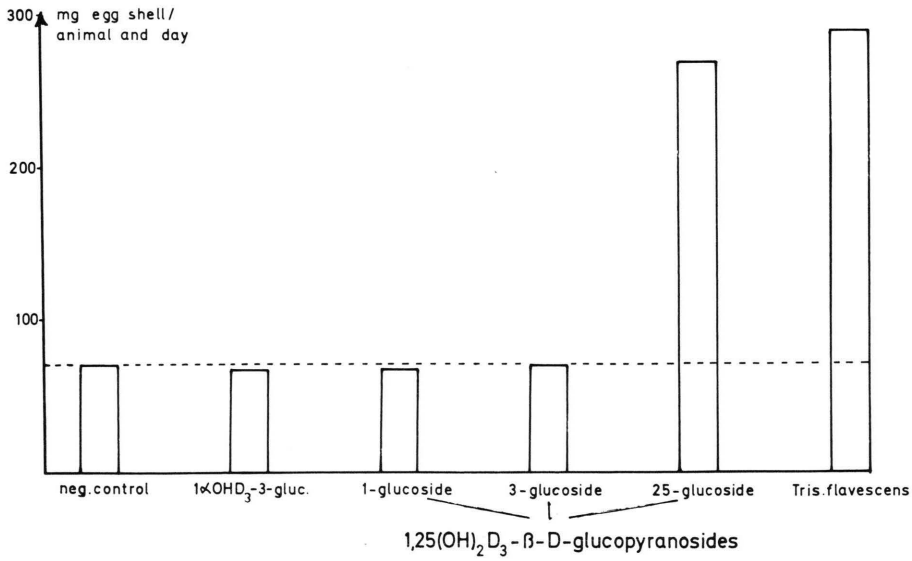

Fig. 1. Japanese quail egg shell test. Effect of different vitamin D metabolite-glucosides (2.8 nmol per kg of diet) on Ca-excretion *via* egg shell. Mean values, n = 10 per group.

Table III. Prophylactic chick assay. $1,25(OH)_2D_3$ as compared to its glucosides. Mean values ± S.D. (n = 14 per group).

Addition in nmol per kg diet	Weight gain [g/day]	Plasma Ca [mg/100 ml]	Bone ash [%]
– (neg. control)	12.3	5.42 ± 0.31	3.95 ± 0.17
1.3 $1,25(OH)_2D_3$-1-gluc.	14.4	5.04 ± 0.62	4.61 ± 0.62
2.6 $1,25(OH)_2D_3$-1-gluc.	17.4	5.14 ± 1.08	4.94 ± 0.62
5.2 $1,25(OH)_2D_3$-1-gluc.	27.7	5.54 ± 1.05	6.55 ± 1.16
1.3 $1,25(OH)_2D_3$-3-gluc.	12.4	4.99 ± 0.85	4.21 ± 0.74
2.6 $1,25(OH)_2D_3$-3-gluc.	16.2	5.23 ± 1.05	4.51 ± 0.89
5.2 $1,25(OH)_2D_3$-3-gluc.	24.0	6.00 ± 0.85	5.99 ± 1.51
1.3 $1,25(OH)_2D_3$-25-gluc.	15.5	4.99 ± 0.43	4.50 ± 0.50
2.6 $1,25(OH)_2D_3$-25-gluc.	24.0	4.95 ± 1.32	5.40 ± 0.43
5.2 $1,25(OH)_2D_3$-25-gluc.	31.9	6.55 ± 0.58	7.56 ± 0.50
1.3 $1,25(OH)_2D_3$	22.0	5.64 ± 1.16	5.29 ± 0.46
2.6 $1,25(OH)_2D_3$	31.2	6.73 ± 0.89	7.11 ± 0.54
5.2 $1,25(OH)_2D_3$	32.0	7.33 ± 0.85	8.12 ± 0.46

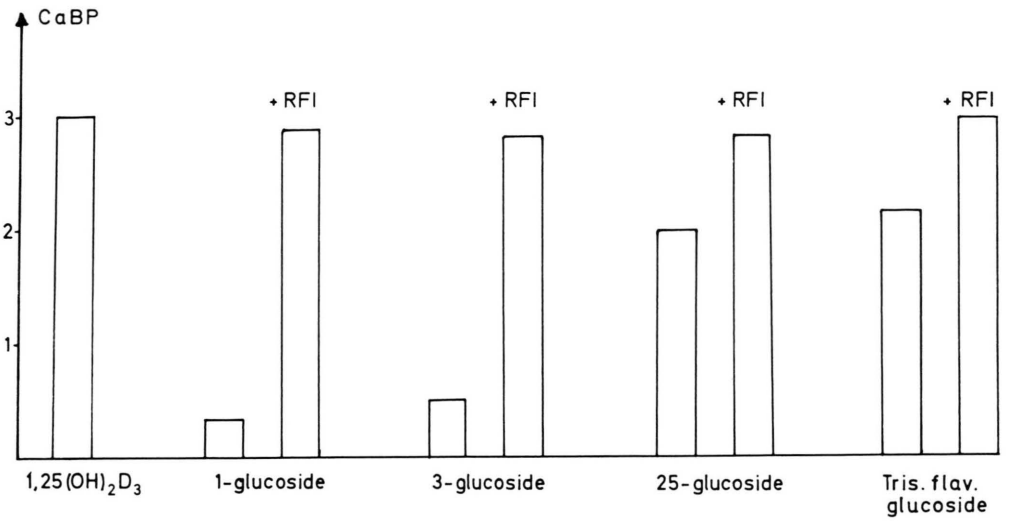

Fig. 2. Synthesis of duodenal CaBP in rachitic chicks. Curative assay. Influence of ruminal fluid incubation (RFI). 2.6 nmol $1,25(OH)_2D_3$-glucosides or 200 g *T. flavescens* (diethyl ether extract) per kg of diet. Mean values from 10 animals per group. CaBP is expressed as described in Material and Methods.

1-glucoside and the 3-glucoside show no vitamin D activity. 1.8 nmol of the $1,25(OH)_2D_3$-25-glucoside, however, increase egg shell production to a similar extent as 400 g *T. flavescens* (residue of diethyl ether extraction).

A more detailed investigation of the bioactivity of the three isomers of $1,25(OH)_2D_3$ glucoside was performed in the prophylactic chick assay (Table III). From weight gain, plasma calcium and per cent bone ash it can be estimated that the 25-glucoside is more than half as active as $1,25(OH)_2D_3$, while the 1- and 3-glucoside showed a much smaller antirachitic potency. While a maximum was reached with 2.6 nmol of $1,25(OH)_2D_3$, 166 nmol of the 1- and 3-glucoside were necessary to obtain similar bone weights [31]. The unexpectedly high bioactivity of the 25-glucoside did not result from an increased hydrolysis of the conjugate in the intestine, since intravenous injection of the three glucosides resulted in the same superiority of the 25-glucoside [32]. Furthermore, the binding constant of the 25-glucoside to the intestinal $1,25(OH)_2D_3$-receptor is one to two orders of magnitude higher than that of two other glucosides [32].

In Fig. 2 the bioactivity of the three glucosides is shown in comparison to the *T. flavescens* glucoside with and without ruminal fluid incubation. Ruminal fluid hydrolyses the glucosides and releases the free steroid hormone with the higher biological activity. From this increase in CaBP it becomes clear that neither the C-1- nor the C-3-glucoside can be the vitamin D metabolite of the grass. Cleavage of the sugar moiety increases their bioactivity at least 10 times. Only $1,25(OH)_2D_3$-25-glucoside is more than half as active as $1,25(OH)_2D_3$ and ruminal fluid potentiates it to the same extent as the *T. flavescens* glucoside. Apart from the high antirachitic activity, the solubility of the 25-glucoside in polar solvents may be of interest for pharmaceutical preparations of this vitamin D_3 metabolite [33].

In conclusion our results indicate that in *Trisetum flavescens* $1,25(OH)_2D_3$ occurs as a glucoside bound *via* carbon 25. This $1,25(OH)_2D_3$-25-glucoside, or rather the $1,25(OH)_2D_3$ liberated by ruminal fluid, can be regarded as the calcinogenic factor of *Trisetum flavescens*. Nothing is known about a possible function of this steroid hormone in the calcinogenic plant.

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