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Degradation chemistry of a Vitamin D analogue (ecalcidene) investigated by HPLC–MS, HPLC–NMR and chemical derivatization

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Abstract

Ecalcidene (1-[(1α,3β,5*Z*,7*E*,20*S*)-1,3-dihydroxy-24-oxo-9,10-secochola-5,7,10(19)-trien-24-yl]-piperidine) is a new 1-hydroxyvitamin D analogue. In this report, the thermal degradation, acid induced degradation and iodine induced degradation of ecalcidene were investigated using HPLC–MS, HPLC–NMR and chemical derivatization. In solution ecalcidene was thermally and reversibly transformed to a pre-Vitamin D type isomer 1 which subsequently produced the dehydrated pyrocalciferol and isopyrocalciferol type isomers 2 and 3 by cyclization and dehydration at elevated temperatures. Acidic conditions resulted in the formation of a novel C_9-hydroxylated isomer 4 of ecalcidene, possibly via a tachysterol type intermediate, followed by the acid facilitated nucleophilic addition of water. In the presence of iodine, *cis*/*trans* isomerization of both ecalcidene and its pre-Vitamin D type isomer 1 occurred. The results may shed light on the stability and metabolism of ecalcidene, provide useful information for its potential pharmaceutical development, and enrich the knowledge of Vitamin D chemistry.

Keywords: Ecalcidene; Degradation; Hydroxylated Vitamin D; HPLC–MS; HPLC–NMR

1. Introduction

Vitamin D is important for many biological processes in higher animals [1–4]. It plays a critical role in the control of calcium and phosphate metabolism, assists in the formation of bone and may also have roles in the control of muscle activity. Vitamin D can prevent myopathy, rickets, osteomalacia and other diseases. Vitamin D itself is a prohormone that requires two in vivo sequential hydroxylations before manifesting optimal physiological activity [5]. The first occurs in the liver to produce 25-hydroxyvitamin D which is the major circulating metabolite of Vitamin D. The second hydroxylation occurs in the kidney and is controlled directly or indirectly by blood calcium and phosphate levels. The product, 1,25-dihydroxyvitamin D is the hormonally active form of Vitamin D and functions as a classical steroid hormone to induce its physiological effects, intestinal calcium absorption and bone calcium mobilization via genomic mechanism. 1,25-Dihydroxyvitamin D also promotes normal cell differentiation and proliferation as well as evokes a variety of biological responses through a non-genomic mechanism. It is well-established that the 1-hydroxy group is necessary for the biological activities of 1,25-dihydroxyvitamin D_3 [6]. The elucidation of the metabolism of Vitamin D has led to a change in emphasis from the study of the parent Vitamin D itself to the study to its metabolites. It also led to a renewed interest in Vitamin D research with regard to its potential use as a therapeutic agent, as in the treatment of cancer and skin disorders such as psoriasis [7–9]. The use of 1,25-dihydroxyvitamin D for the treatment of malignancy resulted in toxic hypercalcemia and stimulated research to find analogues with high potency in inducing cell differentiation but

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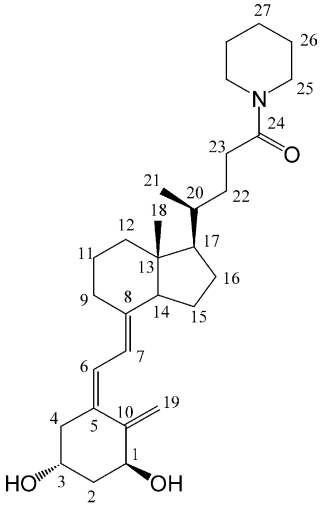
with low calcitropic effects [10,11]. As a matter of fact, many hydroxylated derivatives of Vitamin D have been synthesized to study the structure–activity relationships and to search for compounds showing an increase in specificity or overall activity for potential therapeutic and cosmetic applications. Some of the hydroxylated derivatives are already in development or being used therapeutically [12]. As an example, more than 10 analogues of 1-hydroxylvitamin D have already been approved by governmental agencies or are currently under development by various industrial and/or university research groups for the therapeutic indications of psoriasis, osteoporosis, hypocalcemia, hypoparathyroidism, leukemia, cancer and immune diseases [13]. Ecalcidene (1-[(1α,3β,5*Z*,7*E*,20*S*)-1,3-dihydroxy-24-oxo-9,10-secochola-5,7,10(19)-trien-24-yl]-piperidine), as a new 1-hydroxylvitamin D analogue, was introduced by Hesse et al. and has the potential to serve as a cell modulator [14]. Understanding the degradation properties of ecalcidene is necessary for the development of its potential pharmaceutical applications. In this report, the thermal degradation, acid induced degradation and iodine induced degradation of ecalcidene were investigated. The degradation products were identified using the combination of HPLC–UV, HPLC–MS, HPLC–NMR and chemical derivatization. The degradation mechanisms of ecalcidene induced by temperature, acid and iodine are proposed. Some new chemical features regarding the reactions of this secosteroid 1-hydroxyvitamin D analogue are reported for the first time.



Ecalcidene

2. Experimental

2.1. Chemicals

Ecalcidene and *epi-trans* ecalcidene were obtained from the Laboratory of Process Research at the "Organisch-Chemisches Institut" of the University of Zurich (Switzerland). HPLC grade acetonitrile, phosphoric acid (80%), sodium hydroxide (ACS reagent) and acetic acid (ACS reagent) were obtained from J.T. Baker (Phillipsburg, NJ, USA), maleic anhydride (99%), dimethyl sulfoxide (99.9%, ACS reagent), iodine (ACS reagent) were obtained from Aldrich (St. Louis, MO, USA). HPLC grade water was obtained from Burdick & Jackson (Muskegon, MI, USA). Concentrated ammonium hydroxide was obtained from Mallinckrodt (Paris, KY, USA).

2.2. HPLC–UV analysis

HPLC was performed using an Agilent 1100 HPLC system with diode array UV detection at 267 nm. The UV spectra of the HPLC peaks were obtained using the on-line diode array detector. One analytical HPLC method (Method I) and one semi-preparative HPLC method (Method II) were used. Mobile phase A was prepared by adding 11.4 mL of acetic acid into 4 L of water followed by adjustment of the pH to 4.8 with concentrated ammonium hydroxide. For Method I, a Prodigy ODS (3) column was used (15 cm × 0.32 cm, 3 μm, Phenomenex, Torrance, CA, USA). A binary linear gradient elution using mobile phase A and acetonitrile was conducted: 1–5 min, 50% acetonitrile; 5–7 min, linear gradient to 55% acetonitrile; 7–25 min, 55% acetonitrile at a flow rate of 0.6 mL/min. The injection volume was 10 μL. For Method II, the conditions are the same as for Method I except that a column of the same type with a larger diameter and larger particle size (15 cm × 1 cm, 5 μm) was used; accordingly, the flow rate was scaled up to 4.8 mL/min and the injection volume was 100–1000 μL. The column temperature was 40 °C for both Methods I and II.

2.3. HPLC–MS and direct infusion MS analyses

HPLC–MS analysis was performed on a ThermoFinnigan (San Jose, CA, USA) LCQ mass spectrometer with an atmospheric pressure chemical ionization (APCI) source and a quadruple ion trap mass analyzer in the positive ion mode. The nebulization temperature was 450 °C. The discharge current was kept at 4.9 μA. The heated capillary was kept at 150 °C. The mass spectrometer was interfaced to a TSP HPLC system which consisted of a vacuum degasser, a P4000 pump, an AS3000 autosampler and a UV6000LP PDA detector. The HPLC conditions were the same as for Method I described in Section 2.2. Direct infusion electrospray ionization (ESI) mass spectrometric analysis was performed on a ThermoFinnigan MAT-900 instrument (San Jose, CA, USA) in the positive ion mode. The sample solution was directly infused into the ESI source at a rate of 1 μL/min.

2.4. HPLC–NMR and off-line NMR analyses

HPLC–NMR measurements were carried out on a Varian UnityInova 500 MHz NMR spectrometer (Palo Alto, CA, USA). The spectrometer was equipped with a 60 μL flow probe. The spectrometer was interfaced to a HPLC system. The HPLC conditions were the same as for Method I described in Section 2.2 except that the HPLC column was at room temperature. Also mobile phase A was prepared using D_2O and mobile phase B was prepared using acetonitrile-d_3. Off-line NMR measurements were performed on the Varian UnityPlus 500 MHz

NMR spectrometer (Palo Alto, CA, USA) using CD_3OD as the solvent.

3. Results and discussion

3.1. Thermal degradation

The thermal degradation of ecalcidene was performed by incubating solutions of ecalcidene in dimethyl sulfoxide (DMSO, 0.35 mg/mL) in sealed glass containers for 0.5 h at 25, 100, 140, 145, 150 and 155 °C. The HPLC–UV chromatograms of the reaction solutions are shown in Fig. 1. Ecalcidene eluted at about 13 min. At temperatures of 140 °C or lower, compound 1 was observed as the major degradation product. At temperatures higher than 140 °C, two additional degradation products 2 and 3 were generated along with some other minor species. This report will focus on 1, 2 and 3. Fig. 1 indicates that the yields of 1, 2 and 3 were very dependent on the temperature. The level of ecalcidene decreased rapidly at temperatures greater than 140 °C. Additional HPLC–APCI–MS analyses were performed and the total ion chromatograms (TICs) of the samples exhibited similar profiles to those of the HPLC–UV chromarograms. The UV spectra of peaks ecalcidene, 1, 2 and 3 are presented in Fig. 2(a–d), respectively. HPLC–APCI mass spectrometric studies revealed that, as expected, ecalcidene exhibits ions at m/z 456 $[M+H]^+$, 438 $[M+H-H_2O]^+$ and 420 $[M+H-2H_2O]^+$, which correspond to the protonated molecular ion and the ions losing one or two water molecules. The UV spectrum (Fig. 2(a)) of ecalcidene shows absorption at λ_{max} 267 nm due to the 5,7-diene system. This is consistent with the well-known fact that the

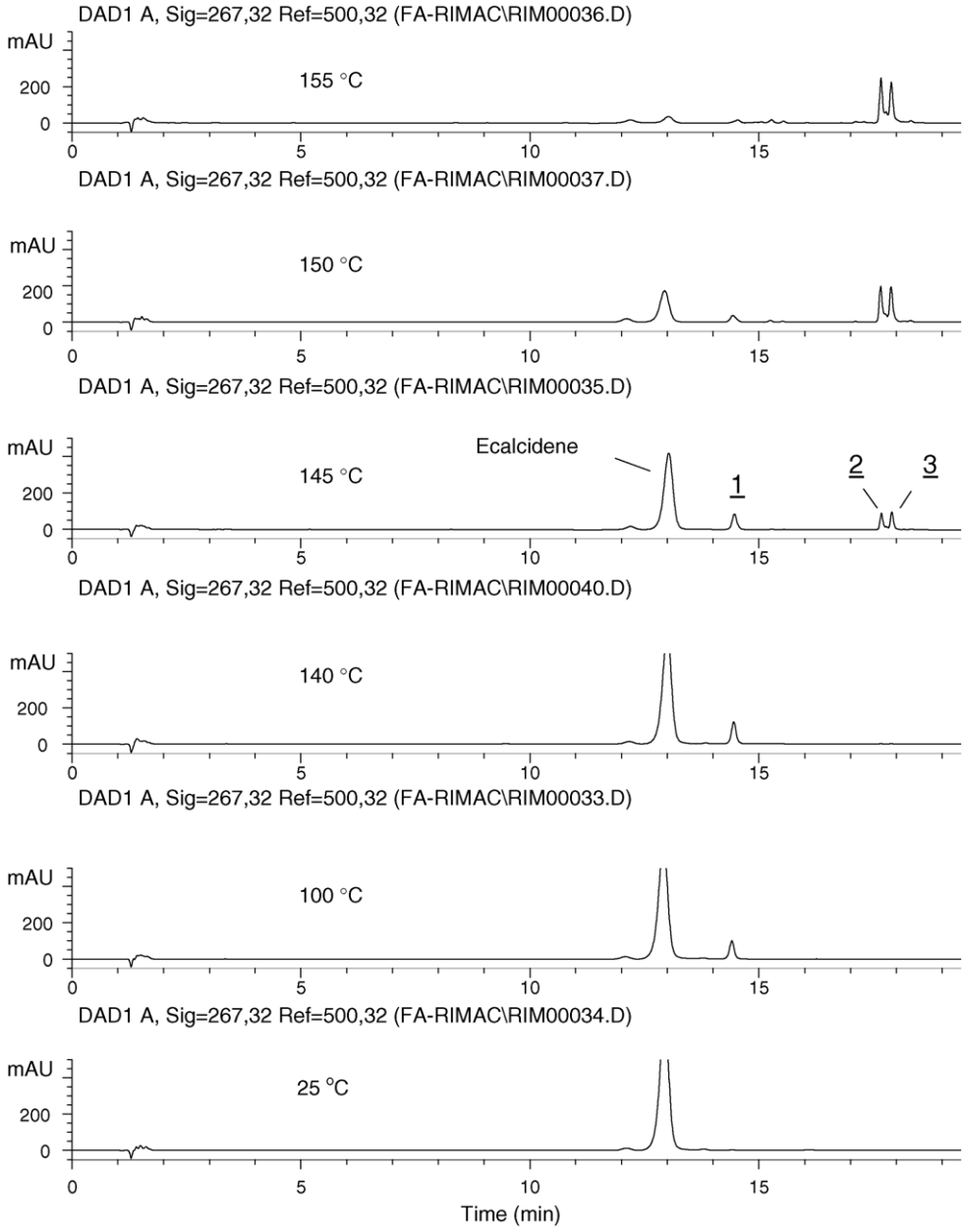


Fig. 1. HPLC–UV chromatograms of ecalcidene in DMSO for 0.5 h.

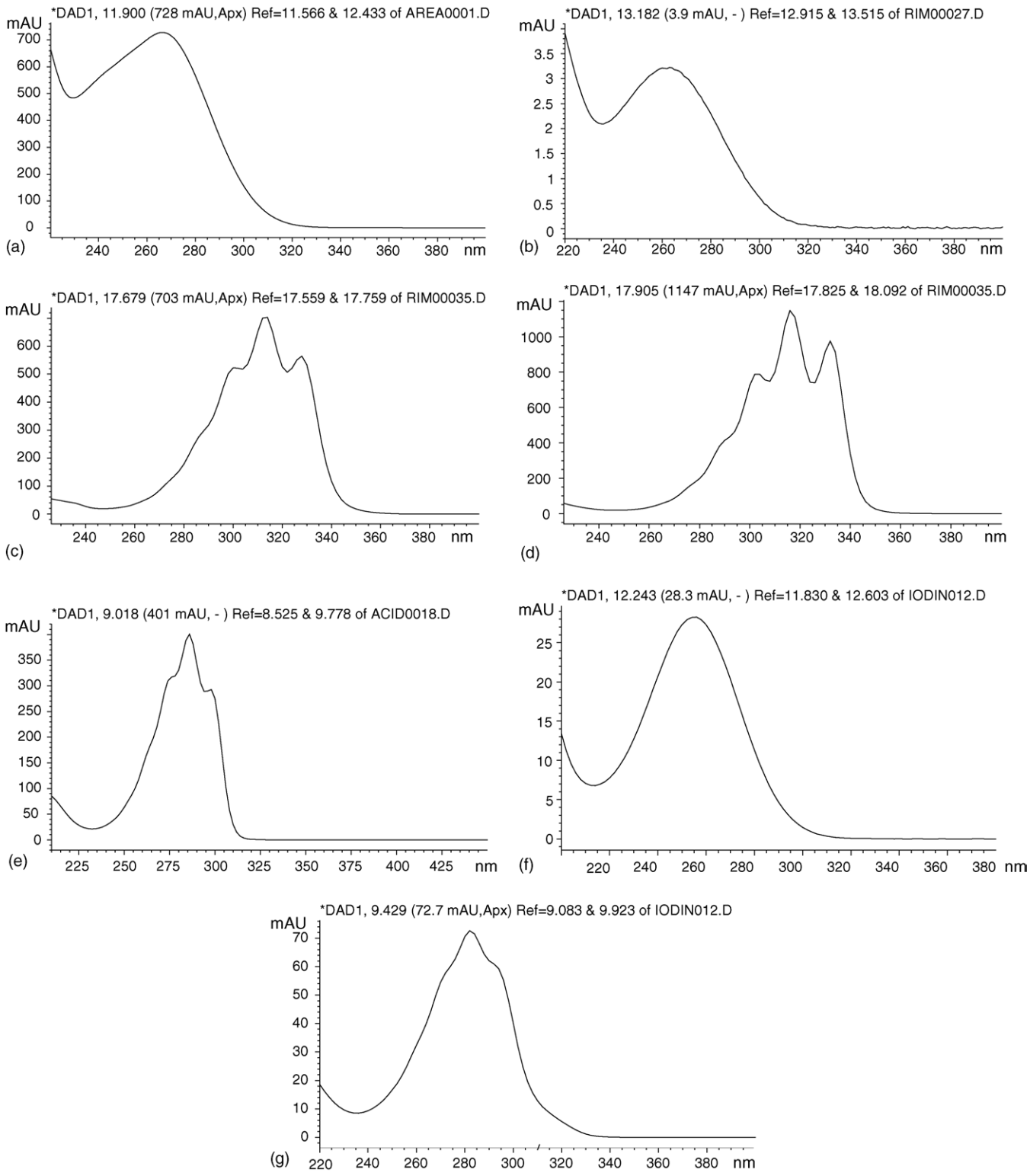
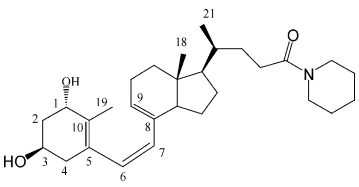
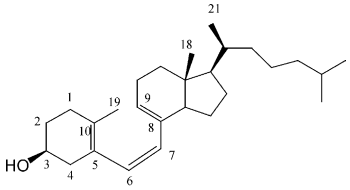


Fig. 2. UV spectra of: (a) ecalcidene, (b) 1, (c) 2, (d) 3, (e) 4, (f) 7 and (g) 9.

5,7-diene nucleus in Vitamin D_2 or D_3 has similar UV absorption at λ_{max} 265 nm [15]. The APCI mass spectrum for degradation product 1 exhibits ions at 456 $[M+H]^+$, 438 $[M+H-H_2O]^+$ and 420 $[M+H-2H_2O]^+$ similar to those of ecalcidene indicating that 1 is an isomer of ecalcidene. To obtain the structural details of 1, HPLC–NMR measurements were performed. The HPLC eluant corresponding to 1 was trapped in the NMR flow probe using a flow–stop technique. For comparative purposes, the HPLC–^{1}H NMR spectrum of ecalcidene was also obtained under the same conditions. By comparing the ^{1}H NMR spectrum of degradation product 1 with solution NMR data of pre-Vitamin D_3 [16], it is proposed that 1 is a pre-Vitamin D type isomer

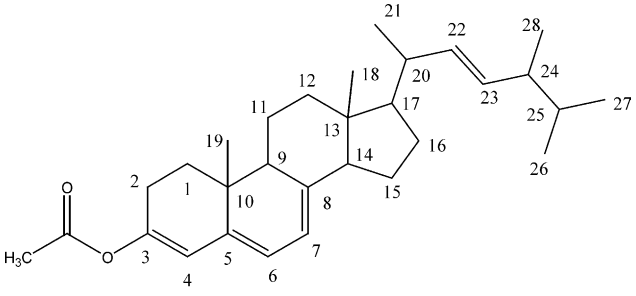
Table 1
Key chemical shifts for 1 and pre-Vitamin D_3

Protons	 **1**	 **Previtamin D_3**	
H1	4.00 (m)		
H3	3.81 (m)		3.85 (m)
H6	5.85 (d, J = 12 Hz)		5.93 (d, J = 11.8 Hz)
H7	5.69 (d, J = 12 Hz)		5.66 (d, J = 11.9 Hz)
H9	5.43 (br s)		5.46 (d, J = 3.5 Hz)
H18	0.62 (s)		0.66 (s)
H19	1.60 (s)		1.61 (s)

of ecalcidene. The key characteristic chemical shifts of 1 and pre-Vitamin D_3 are summarized in Table 1. The only structural difference between 1 and pre-Vitamin D_3 are the 1-hydroxyl and the side chain substitutions. Table 1 shows that both the values of chemical shifts and the coupling patterns of the key protons in 1 correlate quite well to those of pre-Vitamin D_3 and thus indicate their structural similarity. The UV spectrum of 1 (Fig. 2(b)) exhibits a λ_{max} of 263 nm, which has a 4 nm blue shift compared with the absorption of the parent ecalcidene λ_{max} of 267 nm (Fig. 2(a)). Similar phenomena were also observed when comparing the UV absorption of pre-Vitamin D_3 to that of Vitamin D. Pre-Vitamin D and Vitamin D exhibit UV absorptions at 260 and 265 nm, respectively [17], indicating that the postulated structure of 1 is reasonable. The structure of 1 was also confirmed by its unfavorable reactivity towards maleic anhydride, which will be discussed in Section 3.3.

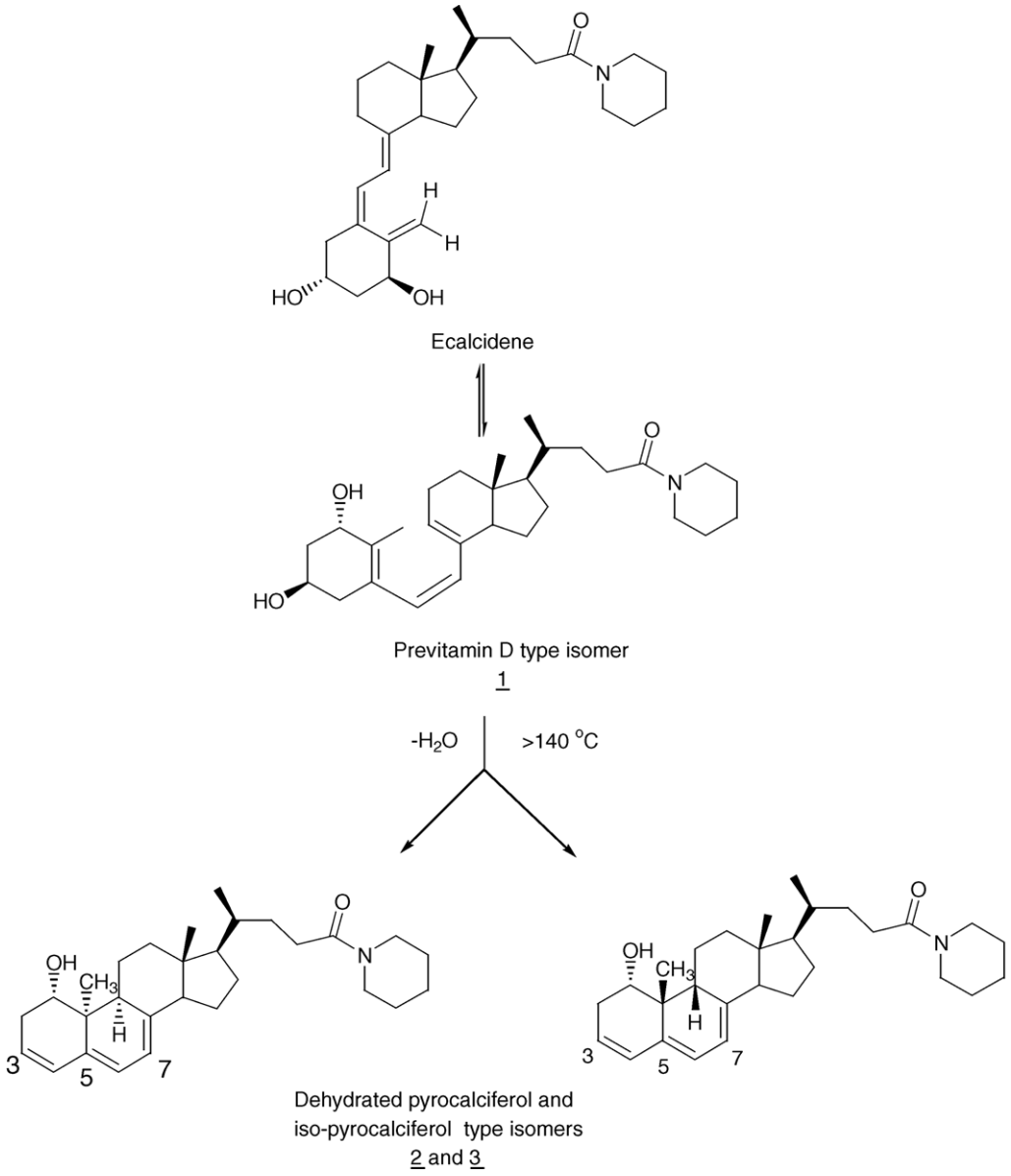
HPLC–NMR experiments indicated that 1 was not stable and can partially revert to the parent ecalcidene in the HPLC mobile phase. Compound 1 was separated by HPLC and trapped in the NMR probe at room temperature followed by periodic ^{1}H NMR measurements. The ^{1}H NMR spectrum of 1 after 4 days of isolation clearly indicates the appearance of the resonances associated with ecalcidene (i.e. the resonances at 6.28 (d, J = 11.2 Hz, H6), 5.96 (d, J = 11.1 Hz, H7), 5.19 (s, H19) and 4.81 (s, H19)). This was confirmed by comparing with the HPLC–^{1}H NMR spectrum of the authentic ecalcidene under the same conditions. In contrast, the ^{1}H NMR spectrum of 1 obtained immediately after HPLC separation did not show the presence of ecalcidene at significant levels.

Both of the APCI mass spectra of 2 and 3 exhibit ions at m/z 438 $[M+H]^+$ and 420 $[M+H-H_2O]^+$. The UV spectra for 2 and 3 are shown in Fig. 2(c and d). Compound 2 exhibits UV absorption peaks at λ_{max} 300, 314 and 328 nm. Compound 3 exhibits UV absorption peaks at λ_{max} 302, 316 and 332 nm. The fact that the UV and MS spectra of 2 is quite similar to 3 suggests that 2 and 3 are a pair of dehydrated isomers of ecalcidene. It has been reported that 3-acetoxyergosta-3,5,7,22-tetraene exhibits UV absorption at λ_{max} 303, 316 and 331 nm [18]. These three UV absorption peaks must be solely contributed by the 3,5,7-triene moiety since the isolated 3-acetoxy group or the isolated double bond at position 22 or the rest of the molecule should have no contribution to the UV spectrum at a wavelength higher than 250 nm according to the well-known Woodward–Fieser rules [19]. The fact that the UV absorption pattern of 3-acetoxyergosta-3,5,7,22-tetraene is quite similar to the UV spectra of 2 and 3 suggests that both 2 and 3 contain the similar 3,5,7-triene moiety. Accordingly, 2 and 3 are proposed to be the dehydrated pyrocalciferol analogue and the dehydrated iso-pyrocalciferol analogue of ecalcidene. Additional work is still needed to distinguish the absolute structures of 2 and 3. The proposed structures and generation mechanism of 1, 2, and 3 are shown in Scheme 1.



3-acetoxyergosta-3,5,7,22-tetraene

Ecalcidene is first reversibly converted to the corresponding pre-Vitamin D type isomer 1 which then generates 2 and 3 by cyclization and dehydration on the B ring at elevated temperatures. The structures of 1, 2 and 3 were proposed based on the aforementioned HPLC–UV, HPLC–MS and HPLC–NMR measurements (Figs. 1 and 2, and Table 1) The reversibility between ecalcidene and 1 was demonstrated by the HPLC–NMR investigation. As presented in Fig. 1, increasing the temperature resulted in the increase of peaks 2 and 3 along with the decrease of the ecalcidene peak. Peak 1 increased at first and then decreased, as would be expected with the mechanism depicted in Scheme 1 wherein 1 behaves as an intermediate generated from ecalcidene and then further reacted to produce the dehydrated



Scheme 1. Proposed thermal degradation mechanism of ecalcidene.

pyrocalciferol analogue 2 and the dehydrated iso-pyrocalciferol analogue 3 at elevated temperature.

It is well-known that in solution Vitamin D_3 can reversibly convert to pre-Vitamin D_3 which can be subsequently rearranged to form pyrocalciferol and iso-pyrocalciferol at elevated temperatures by cyclization of the B ring [20,21]. Both pyrocalciferol and iso-pyrocalciferol exhibit UV absorption at λ_{max} of less than 300 nm [17]. It is not unreasonable to expect that ecalcidene, a close analogue of Vitamin D_3, has a similar thermal degradation pathway accompanied by an additional dehydration process to form a dehydrated pyrocalciferol type isomer and a dehydrated iso-pyrocalciferol type isomer, i.e. 2 and 3, (Scheme 1). The occurrence of dehydration with 1, instead of pre-Vitamin D_3, may be due to the structural difference between ecalcidene and Vitamin D. It is likely that the electron withdrawing 1-hydroxyl group of 1 may facilitate the dehydration of the 3-hydroxyl group to form a favorable triene system. As indicated in Fig. 1, compounds 2 and 3 were generated only at elevated temperature. Prolonged storage of ecalcidene in DMSO at room temperature only yielded 1 as the degradation product.

3.2. Acid induced degradation

The HPLC–UV chromatograms of ecalcidene (0.08 mM/mL) in 1:1 mixture of acetonitrile and 80 mM phosphate buffer with varying pH after 5 days at room temperature are illustrated in Fig. 3. Acetonitrile was used as an inert solvent to increase the solubility of ecalcidene in the aqueous solution. Ecalcidene eluted at about 13 min. At pH 7 and higher, only the pre-Vitamin D type isomer 1 was observed to be the major degradation product. At pH 3, the maximal amount of a new degradation product 4 was formed. Also at pH 1, minor degradation products eluting

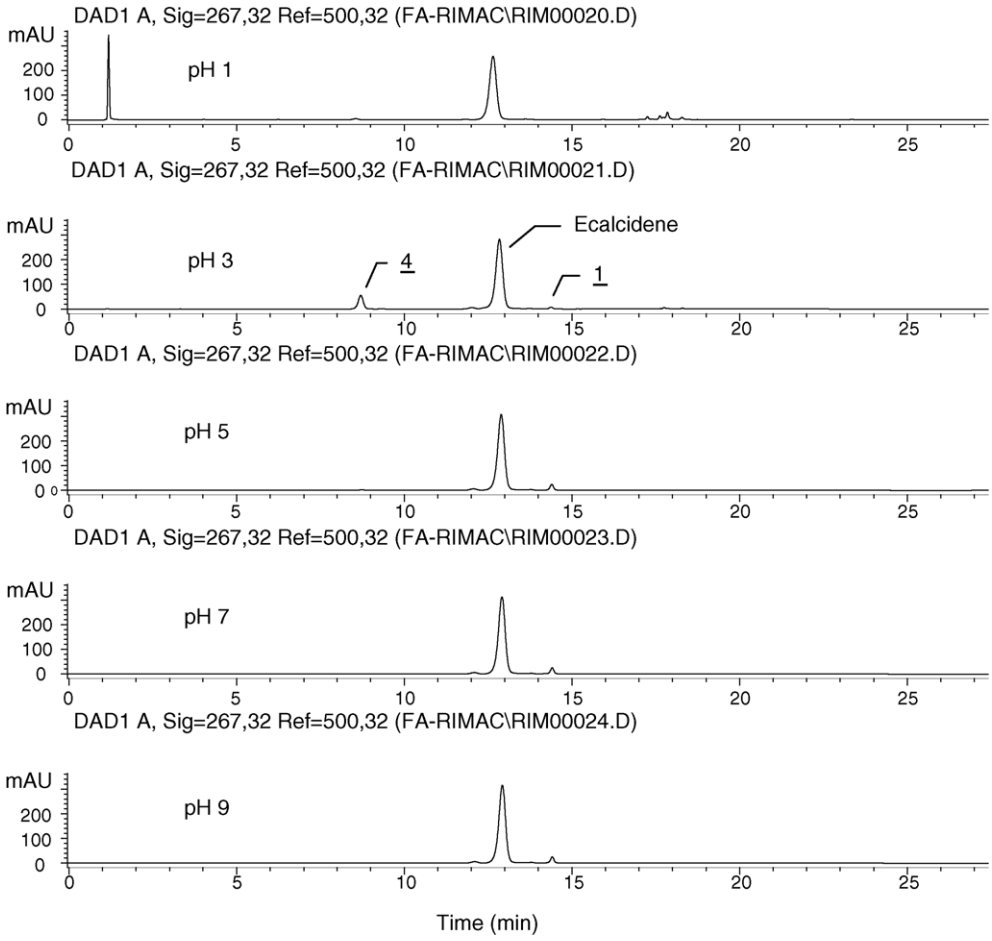


Fig. 3. HPLC–UV chromatograms of ecalcidene at different pH after 5 days at room temperature.

at 17–19 min were observed (the peak at $t_R \sim 1$ min was caused by the buffer). The identity of 1 was confirmed by comparing its HPLC retention time, UV spectrum and APCI mass spectrum with those of 1 as described in Section 3.1. This section will focus on degradation product 4. The UV spectrum of 4 is presented in Fig. 2(e). The APCI mass spectrum of 4 exhibits ions at m/z 438 $[M + H - H_2O]^+$ and 420 $[M + H - 2H_2O]^+$. The molecular weight of 4 was determined to be 455 by direct infusion electrospray ionization (ESI) mass spectrometric analysis described later in this section. The UV spectrum of 4 exhibits overlapped absorption peaks between 250 and 300 nm.

To isolate pure solid of 4 for structural identification, a more concentrated solution of ecalcidene (4.63 mg/mL) in 1:1 mixture of acetonitrile/phosphate buffer of pH 3 was incubated at room temperature for 6 weeks in the dark. HPLC analysis using Method I indicated that 4 is still the major degradation product. This solution (10–1200 μL) was repeatedly injected onto the preparative HPLC column and eluted using HPLC Method II. The eluant corresponding to 4 was collected and the solvent was removed by rotary evaporation under vacuum. The residue was dissolved in acetonitrile and precipitated by the addition of water. The precipitate was collected by centrifugation and dried over P_2O_5 under vacuum to give a white powder. HPLC–UV and HPLC–APCI–MS analyses using Method I revealed that the isolated 4 had a high purity. The isolated 4 was dissolved in the HPLC mobile phase and directly infused into the ESI source of the ThermoFinnigan MAT-900 mass spectrometer to generate ions at m/z 519 $[M + Na + CH_3CN]^+$, 497 $[M + H + CH_3CN]^+$, 478 $[M + Na]^+$, 456 $[M + H]^+$, 438 $[M + H - H_2O]^+$, 420 $[M + H - 2H_2O]^+$, indicating that 4 has a molecular weight of 455 and is an isomer of ecalcidene. In contrast, the HPLC–APCI–MS spectrum of 4 only exhibits ions at m/z 438 $[M + H - H_2O]^+$ and 420 $[M + H - 2H_2O]^+$ without the protonated molecular ion at m/z 456. This is possibly due to the high temperature involved in the APCI vaporization process leading to the dehydration of 4. Additional structural information of 4 was obtained by NMR studies including ^1H, ^{13}C and a series of 2D measurements using CD_3OD as the solvent. The ^1H and ^{13}C NMR data and the assignments of the chemical shifts are presented in Table 2. Compound 4 was identified to be an isomer of ecalcidene with C_1–C_9 hydroxyl migration. The structure of 4 and a postulated generation mechanism are presented in Scheme 2. The absolute configuration at C_9 remains to be determined. As postulated in Scheme 2, the pre-Vitamin D type isomer 1 might be generated first from ecalcidene and subsequently converted to the tachysterol type isomer 5. Acid facilitates the dehydration on C_1 of 5 to yield the cation 6 to which a nucleophilic addition of H_2O may occur to produce

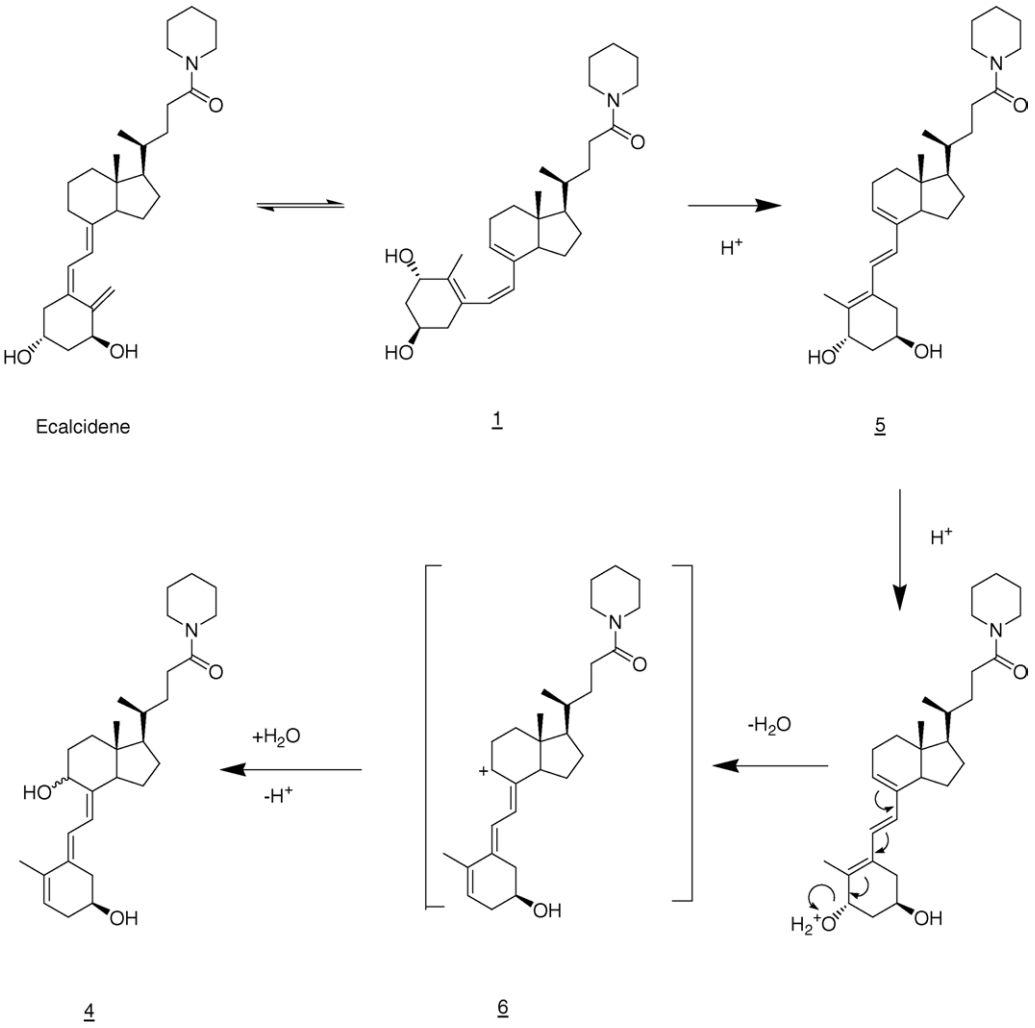
Table 2
NMR resonances of 4

Assignment	Type	δ_C[a]	δ_H[b]	Relative intensity[c]
1	CH	125.9	5.58 (br s)	1
2	CH_2	36.1	2.09, 2.40	2
3	CH	68.1	3.82	1
4	CH_2	35.9	2.21, 2.90	2
5	C	136.4	NA	NA
6	CH	119.6	6.50 (d, J = 11.4 Hz)	1
7	CH	120.4	6.08 (d, J = 11.4 Hz)	1
8	C	144.5	NA	NA
9	CH	65.4	4.87 (s)	1
10	C	135.4	NA	NA
11[d]	CH_2	31.6 or 31.5	1.77	2
12	CH_2	36.3	1.77	2
13	C	47.0	NA	NA
14	CH	51.8	2.65	1
15	CH_2	23.3	1.55	2
16	CH_2	28.5	1.39, 1.94	2
17	CH	57.6	1.43	1
18	CH_3	12.1	0.59 (s)	3
19	CH_3	19.9	1.85 (s)	3
20	CH	36.8	1.54	1
21	CH_3	19.0	0.92 (d, J = 6.6 Hz)	3
22	CH_2	32.7	1.40, 1.94	2
23[d]	CH_2	31.5 or 31.6	2.29, 2.43	2
24	C	174.4	NA	NA
25[e]	CH_2	48.3	3.48	2
26[e]	CH_2	27.9	1.58	2
27	CH_2	25.6	1.66	2
28[e]	CH_2	26.9	1.52	2
29[e]	CH_2	44.1	3.52	2

NA, not applicable.
[a] ^{13}C NMR chemical shift data were obtained from a 1D ^{13}C experiment. Internal TMS was used to reference the chemical shift at 0.00 ppm. The error in chemical shift is estimated to be ±0.3 ppm.
[b] ^{1}H NMR chemical shift data was measured from the g-HSQC experiment. Internal TMS was used to reference the chemical shift at 0.00 ppm. The error in chemical shift is estimated to be ±0.02 ppm.
[c] Relative integrated intensities of proton resonances.
[d] The carbon chemical shifts for these two carbon atoms could not be differentiated in the 2D experiments.
[e] Assignments could be interchanged (25 with 29 and 28 with 26).

4 which has been isolated and structurally identified. The C_9-hydroxylated tachysterol type structure of 4 suggested that a tachysterol type intermediate (5) may exist in this acid induced degradation reaction. Alternatively, the dehydration of C_1 and addition of H_2O to C_9 may occur in a concerted manner. Fig. 3 shows that ecalcidene gives only the pre-Vitamin D type isomer 1 as the major product and its level remains the same in the solutions of pH 7 or higher. This indicated that only thermal isomerization of ecalcidene occurred under neutral or basic conditions. This is a property of the general solution chemistry of ecalcidene and is independent of the specific solvent as demonstrated in Section 3.1. It is also consistent with the well-known characteristic reversible thermal isomerization of Vitamin D to pre-Vitamin D [20,21]. When the pH of the solution was decreased to below 5, the level of 1 decreased and degradation product 4 was observed, indicating that 1 further reacted in acidic media and might serve as the precursor of 4. At pH 3, 4 became the major degradation product. Further decreasing the pH to 1 resulted in the disappearance of 4 and 1 along with the formation of other products eluted between 17 and 19 min suggesting that additional reactions of 4 occurred in more acidic solution. This observation is in agreement with the proposed mechanism that 1 is an intermediate for the formation of 4 as depicted in Scheme 2.

The acid induced degradation of Vitamin D is an important aspect of Vitamin D chemistry. However, there are only very limited reports regarding this process. It was reported that Vitamin D, pre-Vitamin D and tachysterol can be converted to isotachysterol in various acidic conditions such as HCl, BF_3 and H_3PO_3 [22–25]. It was reported that 25-hydroxyvitamin D or 24,25-dihydroxyvitamin D could also be transformed into the corresponding isotachysterols by treatment with hydrochloric acid [26]. However, when 1-hydroxyvitamin D analogues were treated with acidic reagents they were either completely destroyed or yielded a number of unknown products [27]. To the best of our knowledge, there have been no reports regarding the degradation products or the reaction mechanisms. It is the first time that in this report a degradation product has been isolated and structurally identified from the acid induced degradation of a 1-hydroxlated Vitamin D analogue. The established



Scheme 2. Proposed acid induced degradation mechanism of ecalcidene.

C_9-hydroxylated tachysterol type structure of 4 (Scheme 2), can be considered as a water trapped tachysterol type intermediate generated during the acid induced degradation. Scheme 2 serves as a new reaction model for Vitamin D and also suggests that acid induced formation of isotachysterol from Vitamin D may sequentially proceed through the intermediates of pre-Vitamin D and tachysterol.

3.3. Iodine induced degradation

Iodine is an important element in biological systems. Iodine plays a central role in thyroid physiology, being both a major constituent of thyroid hormones and a regulator of thyroid gland function [28]. Iodine disorders induce biological and/or clinical expressions of thyroid dysfunction, and in some cases can disclose pre-existent thyroid abnormalities. Investigation of the interaction between iodine and ecalcidene may shed light on the metabolism and toxicity of ecalcidene for its potential therapeutic application. Similar to Vitamin D_3, ecalcidene has a *cis* configuration in terms of its triene system. In this report, the iodine induced degradation of ecalcidene and its pre-Vitamin D type isomer 1 was studied using HPLC–UV and HPLC–APCI–MS.

3.3.1. Cis/trans isomerization of ecalcidene

The iodine induced degradation of ecalcidene was performed by addition of iodine (25 μg/mL) into a solution of ecalcidene (0.21 mg/mL) in DMSO. The reaction solution was incubated at room temperature for more than 30 min and then monitored using HPLC Method I. The HPLC–UV chromatogram (Fig. 4(a)) of the solution of ecalcidene without iodine exhibits only the peak of ecalcidene. The addition of iodine to the ecalcidene solution resulted in the formation of the new product 7 (Fig. 4(b)). The UV spectrum (Fig. 2(f)) of 7 shows absorption at λ_{max} 275 nm. The APCI mass spectrum of 7 gave a predominant ion at m/z 456 $[M+H]^+$ indicating that 7 is an isomer of ecalcidene. The HPLC–^{1}H NMR spectrum of 7 was obtained. Compound 7 was identified to be the *epi-trans* isomer of ecalcidene by comparing the HPLC–^{1}H NMR data of 7 with NMR data of *epi-trans* Vitamin D_3 [29]. The key chemical shifts of 7 and *epi-trans* Vitamin D_3 are summarized in Table 3. The chemical shifts and coupling patterns of 7 and *epi-trans*

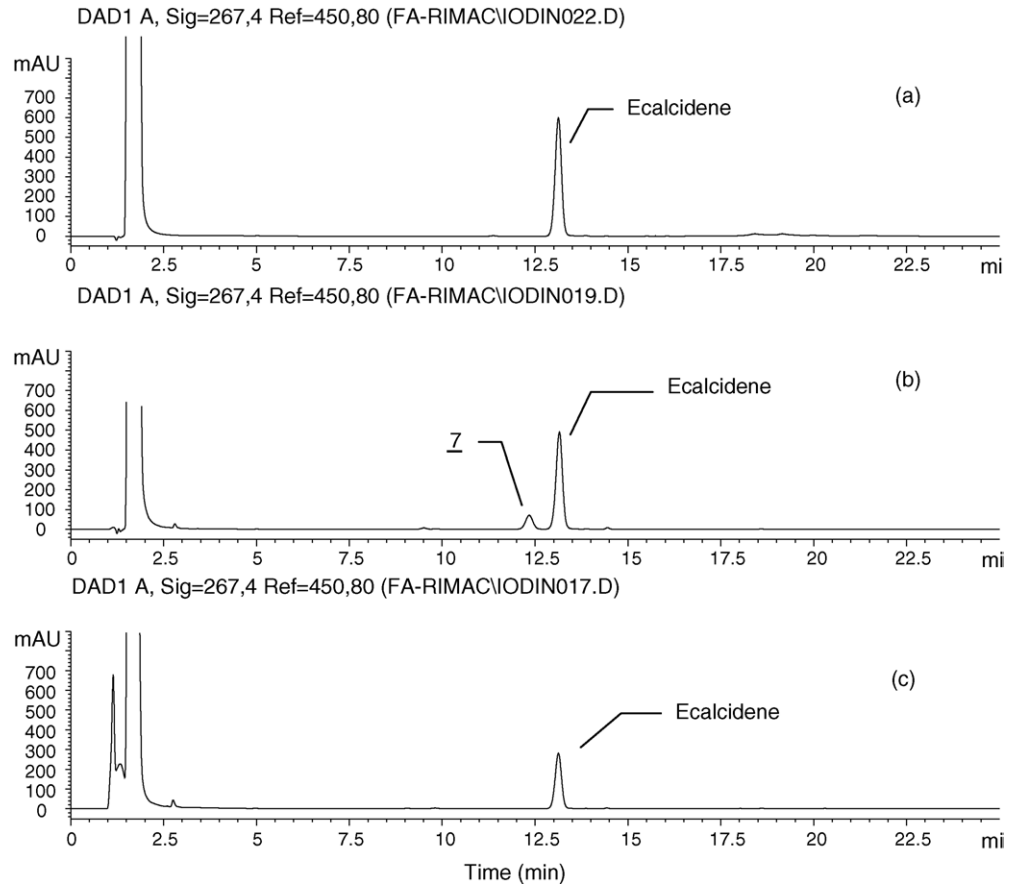


Fig. 4. HPLC–UV chromatograms of (a) ecalcidene, (b) ecalcidene + iodine and (c) ecalcidene + iodine + maleic anhydride.

Vitamin D_3 correlate very well, demonstrating their structural similarity.

It has been reported that *epi-trans* Vitamin D and tachysterol react rapidly with maleic anhydride to form Diels–Alder adducts, while *cis*-Vitamin D and pre-Vitamin D have very slow reaction rates [30]. Accordingly, derivatization with maleic anhydride can be used to distinguish the *epi-trans* Vitamin D and tachysterol forms from the *cis*-Vitamin D and pre-Vitamin D forms. This strategy was used to confirm the structure of 7 as the *epi-trans* isomer of ecalcidene. Fig. 4(c) indicates

Table 3
The key chemical shifts of 7 and *epi-trans* Vitamin D_3

Protons	7	epi-trans vitamin D_3
H6	6.46 (d, J = 11.4 Hz)	6.5 (d, J = 11.1 Hz)
H7	5.83 (d, J = 11.4 Hz)	5.8 (d, J = 11.1 Hz)
H18	0.49 (s)	0.5 (s)
H19	4.86 (s), 4.99 (s)	4.6, 4.9
H21	0.80 (d, J = 6.5 Hz)	

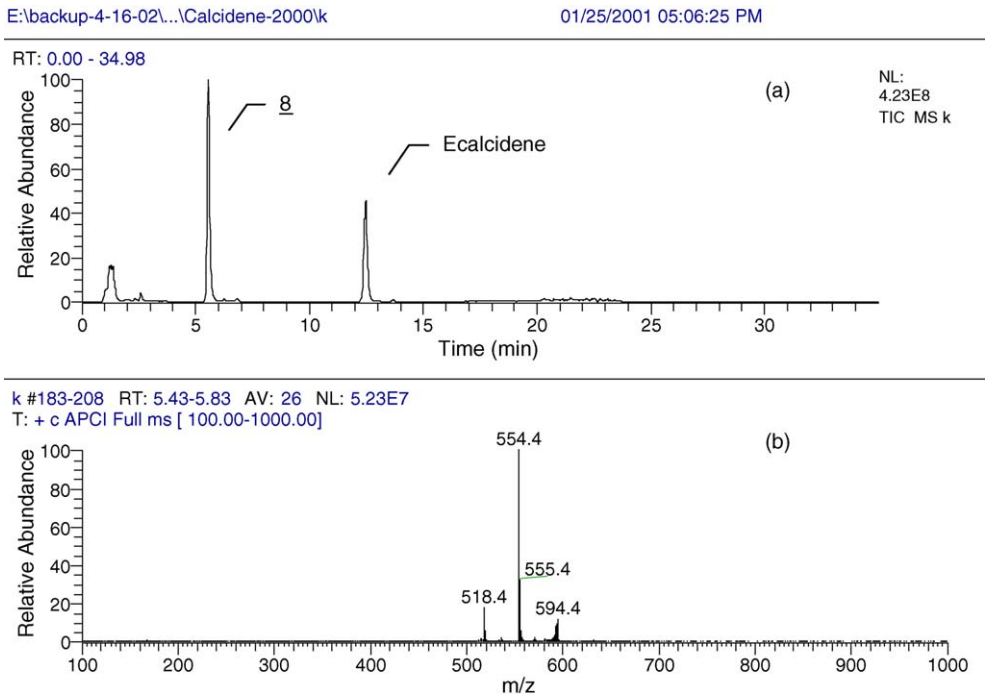
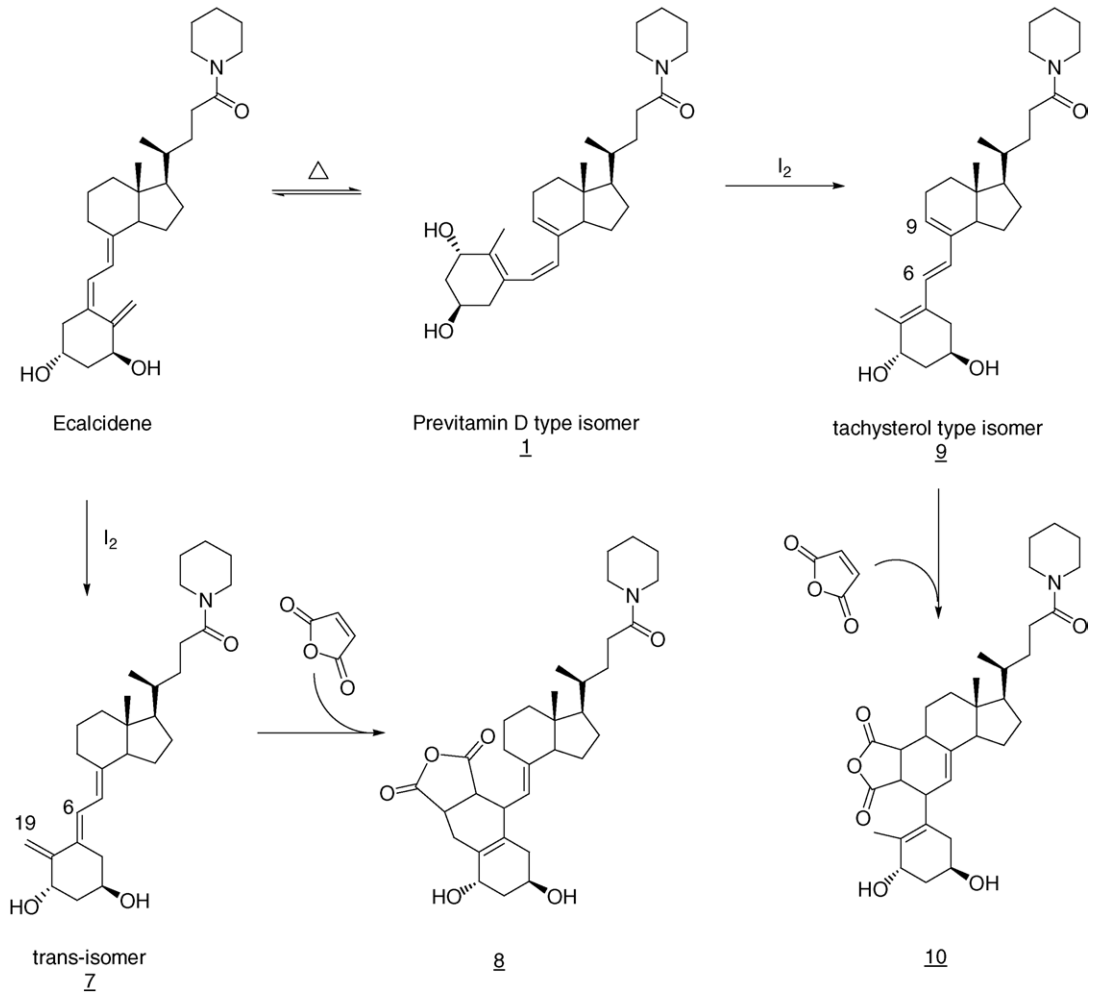


Fig. 5. (a) HPLC–MS chromatogram (TIC) of ecalcidene + iodine + maleic anhydride in DMSO and (b) mass spectrum of adduct 8.



Scheme 3. Proposed iodine induced isomerization pathway of ecalcidene.

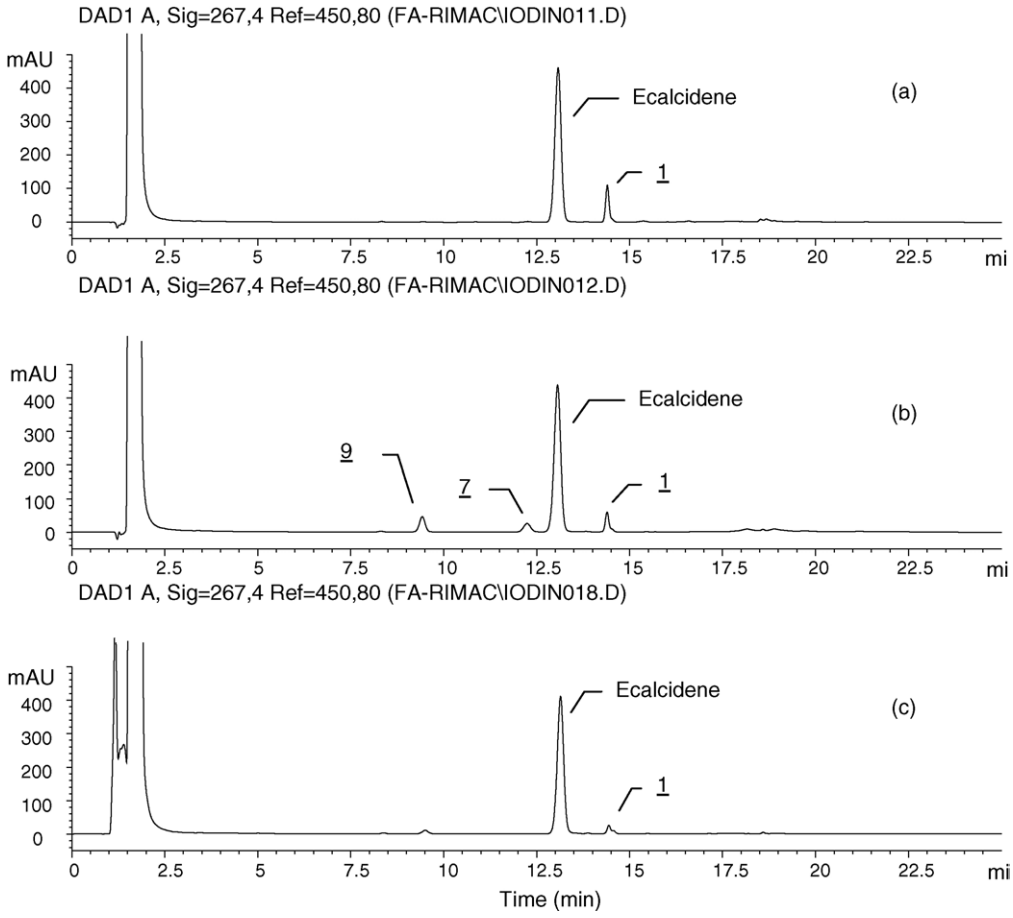


Fig. 6. HPLC–UV chromatograms of (a) ecalcidene at 140 °C for 30 min, (b) ecalcidene at 140 °C for 30 min + iodine and (c) ecalcidene at 140 °C for 30 min + iodine + maleic anhydride.

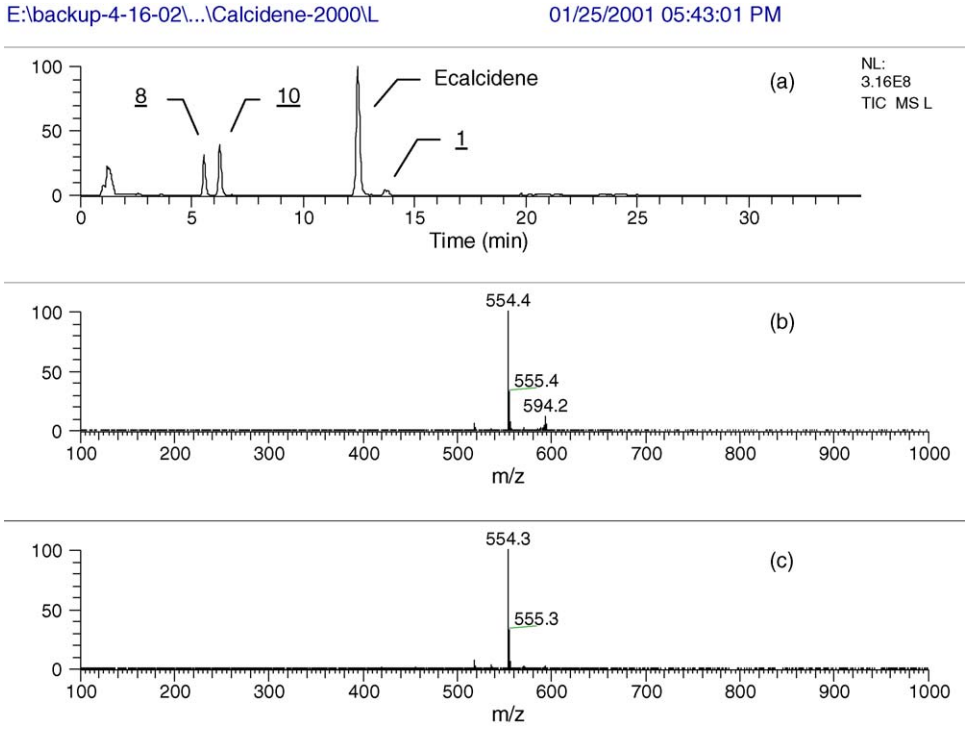


Fig. 7. HPLC–MS chromatogram of (a) ecalcidene in DMSO at 140 °C for 30 min + iodine + maleic anhydride and APCI mass spectra of 8 (b) and 10 (c).

that the formation of 7 was eliminated on addition of maleic anhydride (1.37 mg/mL) to the reaction mixture of ecalcidene (0.21 mg/mL) with iodine (25 μg/mL) in DMSO followed by incubation at room temperature for more than 30 min. The expected Diels–Alder adduct (8) of 7 with maleic acid, was not detected by UV at 267 nm (Fig. 4(c)), but was readily detected by APCI–MS. The total ion chromatogram of this reaction solution is presented in Fig. 5(a). The adduct 8 eluted at ∼5.8 min. The HPLC–APCI–MS spectrum (Fig. 5(b)) of 8 exhibits the expected ions at *m/z* 594 $[M + CH_3CN]^+$, 554 $[M + H]^+$ and 518 $[M + H - 2H_2O]^+$ for a 1:1 adduct of 7 with maleic anhydride. Comparison of the HPLC retention time and the HPLC–APCI–MS spectrum of 7 with that of an authentic sample confirms that 7 is *epi-trans* ecalcidene. Additional HPLC–UV and HPLC–MS experiments demonstrate that the reaction of authentic *epi-trans* ecalcidene with maleic anhydride forms 8. The postulated reaction mechanism is presented in Scheme 3.

3.3.2. Cis/trans isomerization of pre-Vitamin D type isomer 1

The pre-Vitamin D type isomer 1 was generated by thermal degradation of ecalcidene as described in Section 3.1. A solution of ecalcidene (0.21 mg/mL) in DMSO was kept at 140 °C for 30 min. The HPLC–UV chromatogram (Method I) of this reaction solution is presented in Fig. 6(a) and confirms the formation of 1. Fig. 6(b) shows that the addition of iodine (25 μg/mL) to a solution of ecalcidene containing thermally generated 1 leads to the formation of the *epi-trans* isomer 7 and a new compound 9 after incubation at room temperature for more than 30 min prior to HPLC analysis. Compound 9 must be produced from 1 since ecalcidene only generates 7 in presence of iodine as established in Section 3.3.1. The UV spectrum of 9 is presented in Fig. 2(g). The structure of 9 is proposed to be a tachysterol type isomer of ecalcidene, i.e. a *trans*-isomer of 1. The UV spectrum of 9 exhibits an absorption peak at 281 nm with shoulders at 270 and 296 nm. This is similar to the UV of tachysterol, which is also a *trans*-isomer of pre-Vitamin D, with an absorption peak at 281 nm and shoulders at 272 and 289 nm [17]. The mass spectrum of 9 shows ions at *m/z* 456 $[M + H]^+$, 438 $[M + H - H_2O]^+$ and 420 $[M + H - 2H_2O]^+$ revealing that 9 is an isomer of 1 and ecalcidene. The structure of 9 and a proposed formation pathway are presented in Scheme 3. De Vries et al. reported that besides *epi-trans* Vitamin D, tachysterol also has a high reactivity towards maleic anhydride [30]. The structure of 9 was confirmed by using the derivatization with maleic anhydride, as was used to confirm the structure of 7 (Section 3.3.1). Fig. 6(c) indicates that addition of maleic anhydride to the mixture containing ecalcidene, 7, 1 and 9 eliminated 7 and 9. The reaction of maleic anhydride with 7 and 9 would generate the corresponding adducts 8 and 10. Although 8 and 10 were not detected by UV at 267 nm (Fig. 6(c)), 8 and 10 were readily detected using HPLC–APCI–MS (Fig. 7(a)). As described in Section 3.3.1, 8 was formed from the reaction of 7 with maleic anhydride and accordingly 10 must be formed from the reaction of 9 with maleic anhydride. The mass spectra (Fig. 7(b and c)) of 8 and 10 exhibit ions at *m/z* 594 ($M^+ + CH_3CN$), 554 (MH^+) and 518 ($MH^+ - 2H_2O$), which are consistent with a 1:1 adduct of 7 or 9 with maleic anhydride. The postulated structures of 8 and 10 and formation pathway are presented in Scheme 3. Since the Diels–Alder reaction requires that the conjugated diene system in the dienophile must be in an easily accessible cisoid conformation [31], maleic anhydride added to the C_6 and C_{19} positions of 7 to form 8, but added to the C_6 and C_9 positions of 9 to form 10. By similar reasoning, ecalcidene, Vitamin D, pre-Vitamin D and the pre-Vitamin D type isomer 1 have low reactivity towards maleic anhydride. That ecalcidene, which has a *cis* configuration, and its corresponding pre-Vitamin D type isomer 1 can be converted by iodine to *epi-trans* ecalcidene 7 and the tachysterol type isomer 9 is consistent with the well-known fact that Vitamin D, which also has a *cis* configuration, and pre-Vitamin D can be converted by iodine to the corresponding *epi-trans* Vitamin D and tachysterol [32].

4. Conclusions

The thermal, acid induced and iodine induced degradation of ecalcidene (1-[(1α,3β,5*Z*,7*E*,20*S*)-1,3-dihydroxy-24-oxo-9,10-secochola-5,7,10(19)-trien-24-yl]-piperidine), a 1-hydroxylated analogue of Vitamin D, was studied. Like Vitamin D, ecalcidene has a reversible isomerization to the corresponding pre-Vitamin D type isomer 1 which can be subsequently transformed into the pyro and iso-pyro isomers at elevated temperatures via cyclization of the B ring. However, this was accompanied by an unexpected dehydration of the 3-hydroxyl group. The presence of the 1-hydroxyl moiety may have been responsible for this since the 3-hydroxyl group in Vitamin D remains intact during a similar thermal transformation [20,21]. In aqueous acidic media, ecalcidene underwent a novel C_1–C_9 hydroxyl migration. That this occurred, possibly via a tachysterol type intermediate, would be the first observation of this new type of reaction so far for 1-hydroxyvitamin D analogues. Ecalcidene and its corresponding pre-Vitamin D type isomer can undergo *cis*/*trans* conversion by interaction with iodine, which is similar to the behavior of Vitamin D [32]. A combination of various techniques including on-line HPLC–UV, HPLC–MS, HPLC–NMR, chemical derivatization and sample isolation followed by off-line spectrometric analyses were successfully applied during these studies. These results may provide useful guidelines and a foundation for the potential pharmaceutical development of ecalcidene in terms of stability, metabolism, toxicity, formulation improvement, packaging selection, analytical procedure establishment, etc. The results may also enrich the knowledge of Vitamin D chemistry.

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