Actinomyces hyovaginalis: A novel bacterial isolate with transforming activity of vitamin D_3 to 1α, 25-dihydroxyvitamin D_3

Ahmad M. Abbas; Khaled M. Aboshanab, Mohammad M. Aboulwafa * and Nadia A. Hassouna

Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, Organization of African Unity St., POB: 11566, Abbassia, Cairo, Egypt
*maboulwafa@yahoo.com

Abstract: A total number of 180 bacterial isolated were recovered from various soil samples collected from different localities in Egypt using paraffin baiting technique. A screening program applied on the collected isolates scored five isolates coded A11-2; A13-4; A8-4; A26-7 and A26-8 with potential activity for vitamin D3 transformation. Preliminary analysis, using TLC, showed that the selected isolates were able to transform vitamin D_3 into 1α, 25-dihydroxyvitamin D_3 (calcitriol) with 25-hydroxyvitamin D_3 (calcidiol) as an intermediate. Mass spectrometric (MS/MS) analyses of the produced calcidiol and calcitriol by the respective isolates showed that isolate A11-2 exhibited the highest product intensity of the m/z precursor ions of both calcidiol and calcitriol. The isolate A11-2 was selected and identified using microscopical, culture and biochemical characteristics as well as Biolog microbial identification system as *Actinomyces hyovaginalis*. This is the first report for vitamin D_3 transformation into its biologically active forms by the genus Actinomyces.
[Ahmad M. Abbas; Khaled M. Aboshanab, Mohammad M. Aboulwafa and Nadia A. Hassouna *Actinomyces hyovaginalis*: A novel bacterial isolate with transforming activity of vitamin D_3 to 1α, 25-dihydroxyvitamin D_3. Journal of American Science 2011; 7(9): 230-237]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: vitamin D_3 biotransformation, calcidiol, calcitriol, *Actinomyces hyovaginalis*.

1. Introduction:

Vitamin D_3 is a member of a family of fat-soluble prohormones (McCollum *et al*., 1921). It carries out the vital biological function of maintaining appropriate levels of calcium and phosphate which are needed for the normal mineralization of bone, muscle contraction, nerve conduction, and general cellular function (Bouillon *et al.*, 1995; DeLuca, 2004). Its deficiency can result in impaired bone mineralization, and leads to bone softening diseases such as rickets in children and osteomalacia in adults, and definitely contributes to osteoporosis (Larry, 2007). What unique about vitamin D_3 is that it is biologically inert and to exert its function, it must be first activated by hydroxylation in two sequential steps (DeLuca and Schnoes, 1983), first in liver where 25-hydroxyvitamin D_3 (calcidiol) is produced then further hydroxylation takes place in kidney to produce the fully active 1α, 25-dihydroxy vitamin D_3 (calcitriol). Such hydroxylation steps are mediated by cytochrome P-450 hydroxylase enzymes. Calcitriol exerts the most potent effect on the mobilization of calcium in the process of bone formation (Seino *et al*., 1987); However, its production in patients with liver and/or kidney problems is greatly affected so synthetic calcitriol has been used clinically to treat several disease states such as chronic renal failure, hypo- parathyroidism and osteoporosis (Eastell and Riggs, 1997; Grant and Holick, 2005). Unfortunately, the chemical synthesis of calcitriol especially regio- and stereo- selective introduction of a hydroxyl group at C-1 is a very expensive and tedious procedure requiring many reaction steps (Kametani and Furuyama, 1987). Such criteria have necessitated the need to apply enzymatic chemistry using microorganisms to hydroxylate vitamin D_3 at the 1α- and /or 25- positions (Sasaki *et al*., 1991 and 1992). Microorganisms capable of hydroxylating vitamin D_3 compounds were actually very limited and belong to the order Actinomycetales particularly the two genera *Streptomyces* and *Amycolata* (Sasaki *et al*., 1991 and 1992). Accordingly, the present study aimed at studying biotransformation of vitamin D_3 into calcitriol using bacterial isolates collected from different Egyptian soil samples. In this manuscript a screening program was carried out to isolate a promising bacterial isolate(s) capable of transforming vitamin D_3 into its biologically active forms.

2. Material and Methods

Microorganisms and culture conditions

Microorganisms recovered from various soil samples collected from different localities of Delta, Egypt were cultured on paraffin agar medium at 28°C for 2-5 days. Selective recovery of the genera *Nocardia*, *Amycolata* and *Actinomyces* isolates was carried out via paraffin baiting technique (Gordon and Hagan, 1936). Bacterial colonies appearing as white chalky particles around the solidified paraffin wax globules were collected and examined microscopically. Pure cultures were maintained onto nutrient agar slants at 4°C until screening for vitamin D_3 biotransformation. For stock cultures, cells were

concentrated and suspended in slant medium 50:50 and stored at – 20°C (Miller, 1972).

Basal medium used for vitamin D_3 biotransformation: consisted of 15 g glucose, 15 g defatted soyabean, 5 g sodium chloride, 2 g calcium carbonate, 1 g dipotassium hydrogen phosphate, 0.5 g sodium fluoride per liter distilled water with initial pH of 7.8. (Sasaki *et al*., 1991 and 1992).

Screening procedures for vitamin D_3 biotransformation

Growth conditions and extract preparation

A single colony of the bacterial isolate was transferred to 10 ml nutrient broth (Lab M, Topley house, England) contained in 100-ml Erlenmeyer flask which was incubated in shaking incubator at 28°C and 200 rpm for 2 days (preculture). An aliqout (1 ml) of the preculture obtained was used to inoculate 50 ml basal medium used for vitamin D_3 bioconversion contained in 250-ml Erlenmeyer flask (main culture), incubated at 28°C and 200 rpm for 2 days. Thereafter, 10 mg of vitamin D_3 (Medical Union Pharmaceuticals (MUP), Cairo, Egypt), dissolved in 250 µl 96 % ethanol, was added and the culture was continued, under the same conditions, for another 7 days. Vitamin D_3 and its metabolites were extracted using methylene chloride and methanol (El-Nasr chemical Co. (Adwic), Cairo, Egypt), according to a method described by Bligh and Dyer (1959), with minor modifications as follows.

Fermentation broth was collected in a 50-ml plastic falcon tube and centrifuged at 4000 rpm for 20 min then the supernatant was collected in 250-ml Erlenmeyer flask and was mixed with ammonium sulphate (56 g/100 ml) to give 80% saturation (Foster *et al*., 1971). Then the supernatant was shaken at 140 rpm for 60 min, allowed to equilibrate in refrigerator for 30 min then centrifuged at 4000 rpm for 10 min. The supernatant was collected and mixed with 50 ml of methanol/methylene chloride mixture (2:1), shaken at room temperature at 140 rpm for 40 min. Thereafter, 25 ml of methylene chloride was added and the mixture was shaken for further 40 min. The whole content was transferred to a separating funnel, allowed to stand till complete phase separation. The lower clear organic layer was collected and the remaining aqueous layer was similarly re-extracted as described before but with half volumes of the organic solvents. After phase separation, the organic layers of both extracts were collected in a suitable evaporating flask with neck 19-B. and evaporated under vacuum using rotary evaporator at 50°C. The dried residue obtained was dissolved in 1.5 ml methanol (HPLC grade).

Analyses techniques

TLC analysis

The concentrated extracts of tested isolates (prepared as described above) were preliminarily analyzed by using TLC. Samples (40 µl) of each extract as well as vitamin D_3, 1α-hydroxyvitamin D_3 and calcitriol as standards were applied onto TLC sheets (Merck, F_{254}) and separated using the mobile phase chloroform/methanol (10:1). Detection of spots was performed using UV light at 254 nm wavelength. Since calcidiol was commercially unavailable, 1α-hydroxyvitamin D_3 was used, instead, as a reference for rough detection of calcidiol (the intermediate of vitamin D_3 biotransformation into calcitriol).

MS analysis

The concentrated extracts showed suggestive results for calcitriol production, using TLC analysis were further analyzed using MS and MS/Ms analyses. The assay was performed by the means of electron spray ionization (ESI) in positive ion mode and selective reaction monitoring (SRM). The conditions were as follows: source voltage, 4.52 kV; source current, 2.39 uA; sheath Gas flow rate, 19.24 L/h; aux/sweep gas flow rate, 20 L/h; capillary voltage, 3.36 V; capillary temperature, 200.1°C and tube lens voltage, 55 V.

Isolate identification.

The selected isolate with relative higher activity for vitamin D_3 transformation was identified based on both microscopical, culture, biochemical characteristics according to Gordon *et al*. (1974) and Holt (1994) and Biolog microbial identification system assay.

Biolog microbial identification system assay.

This assay was carried out at microbiological resources center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University and was conducted using the Biolog GP2 MicroPlate (BIOLOG, Inc., Hayward, California, USA) which contains 96 wells, the first well contained water while the remaining wells contained 95 different carbon sources, each in a single well. All wells contained a redox indicator (tetrazolium dye). The assay was done as follows

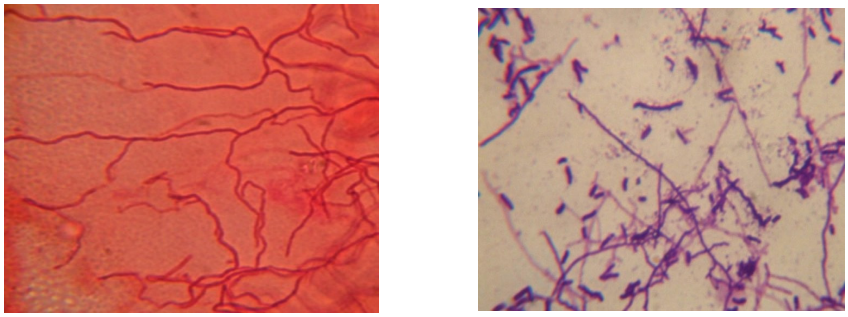
(1) A pure culture of a bacterium was streaked on a Biolog Universal Growth agar plate using "plus" sign streaking technique.
(2) After 24 hrs incubation, the bacterial growth was swabbed from the surface of the agar plate, at the ends of the "Plus" sign to half way down the junction of the two lines constituting the "Plus" sign, and suspended to a specified density (adjust turbidity to match the GP-ROD SB turbidity standard, T= 28% +/- 2%) in GN/GP-IF (Gram negative/gram positive inoculating fluid).

(3) An aliquot (150 µl) of bacterial suspension was pipetted into each well of the GP2 MicroPlate.
(4) The MicroPlate was incubated at 30°C for 24 hours and then, was read with the Biolog MicroStation and compared to the GP database.

3. Results

Recovery of bacterial isolates

A total number of 180 bacterial isolates were recovered. A bacterial colony appeared as white chalky particles around the solidified paraffin wax globules was picked up and examined microscopically (100X). Simple stain revealed delicate irregularly bent short mycelia with or without fragmentation (Fig. 1). Such growth and microscopical characters are suggestive for *Nocardia*, *Amycolata* and *Actinomyces* species.



(a) **(b)**

Fig. 1: Microscopical examination (100x) of an isolate showing delicate irregularly bent mycelia displaying true branching (a) and an isolate showing fragmented mycelia (b).

TLC analsysis

As depicted in Fig. 2. only five isolates coded A11-2, A13-4, A8-4, A26-7 and A26-8 showed two spots with R_f values comparable to those exhibited by 1α-hydroxyvitamin D_3 (R_f = 0.45) and calcitriol standard (R_f=0.25).

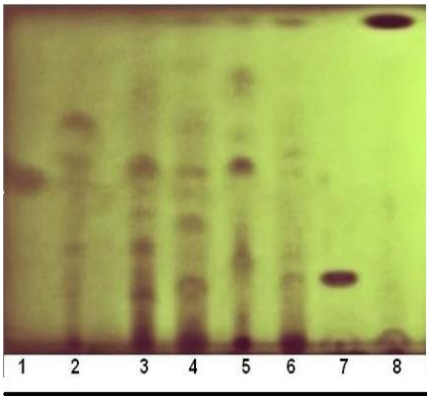


Fig. 2: TLC analysis profile, as photographed under UV light, of concentrated extracts of the scored five soil isolates A26-8, A13-4, A11-2, A26-7, A8-4 (lane 2-6, respectively) against 1α-hydroxyvitamin D_3 (lane 1), calcitriol standard (lane 7) and vitamin D_3 (lane 8).

MS analysis

MS analysis of the concentrated extract of each of the selected five isolates showed precursor ions *m/z* 416, 399, 400 and 383. As a representative example, the MS analysis profile of the concentrated extract of isolate A11-2 is shown in Fig. 3.

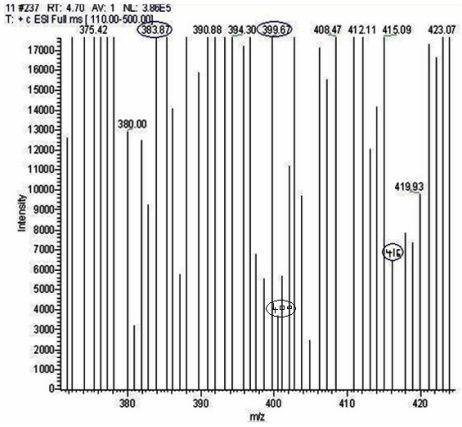


Fig. 3: MS analysis profile of the concentrated extract of isolate A11-2. The two precursor ions of calcitriol (*m/z* 399 and 416) and those of calcidiol (*m/z* 383 and 400) are marked by circles.

MS/MS analysis

MS/Ms analysis was carried out for distinguishing the precursor ion(s) *m/z* 399 and/or 416 in both calcitriol standard preparation and the concentrated extracts of the five selected isolates and for

distinguishing the precursor ion(s) *m/z* 383 and/or 400 in the concentrated extracts of the five selected isolates only. MS/MS for calcitriol standard showed one precursor ion *m/z* 416 while the concentrated extracts of the five selected isolates showed two precursor ions *m/z* 399 and 416 (characteristic for calcitriol) and another two precursor ions *m/z* 383 and 400 (characteristic for calcidiol) (Fig. 4).

As a representative example, the MS/MS profile of the concentrated extract of isolate A11-2 is shown in Fig. 4. Precursor ions detected by MS/MS in different preparations were subjected to increased collision energy values for characterizing their fragmentation patterns.

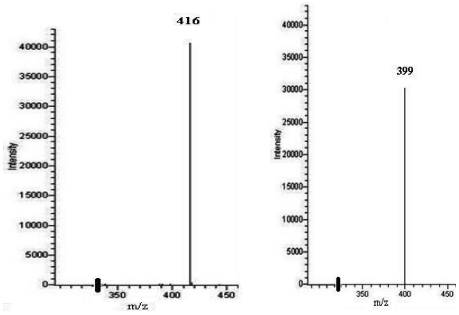
(i) Calcitriol precursor ions (*m/z* 416 and/or 399).

a) Precursor ion *m/z* 416 was detected in both calcitriol standard preparation and in the concentrated extract of the isolate A11-2. Fragmentation of this ion resulted in the appearance of a characteristic fragment ion *m/z* 343 in both standard and tested preparations (Fig. 5). A remaining low intensity non-fragmented portion of the precursor ion *m/z* 416 was present only with the standard calcitriol preparation. However, other additional fragment ions of different *m/z* values were detected with the tested preparation.

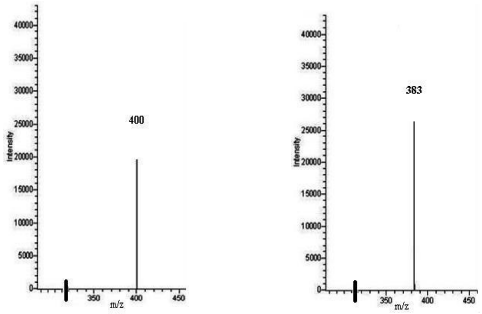
b) Precursor ion *m/z* 399 was detected only in the tested preparation. Its fragmentation resulted in the appearance of a characteristic fragment ion *m/z* 381 together with a remaining non-fragmented portion and other non characteristic fragment ions (Fig 6).

ii) Calcidiol precursor ions (*m/z* 400 and/or 383).

For the precursor ion *m/z* 400, which was detected in the test preparations, no fragment ions appeared upon raising the collision energy. For the precursor ion *m/z* 383, the fragmentation resulted in the appearance of a characteristic fragment ion *m/z* 365 together with non-fragmented portion and other non-characteristic fragment ions (Fig 7).

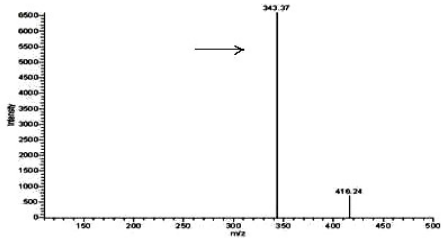


(a)

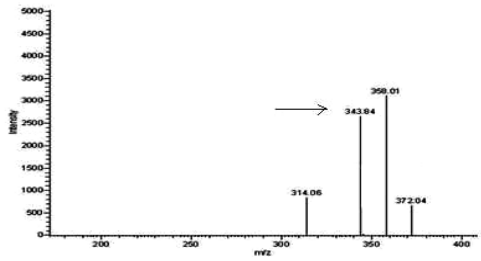


(b)

Fig. 4: MS/MS profile of the concentrated extract of isolate A11-2 showing precursor ions characteristic for calcitriol (a) and calcidiol (b).



(a)



(b)

Fig. 5: Fragmentation pattern of the precursor ion *m/z* 416 of the standard calcitriol preparation (a) and the concentrated extract of isolate A11-2 (b). The fragment ion *m/z* 343, appearing in both standard and test preparations, is marked by arrow.

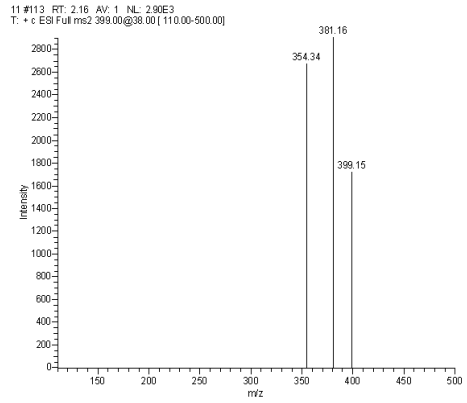


Fig. 6: Fragmentation pattern of the precursor ion *m/z* 399 of the concentrated extract of isolate A11-2.

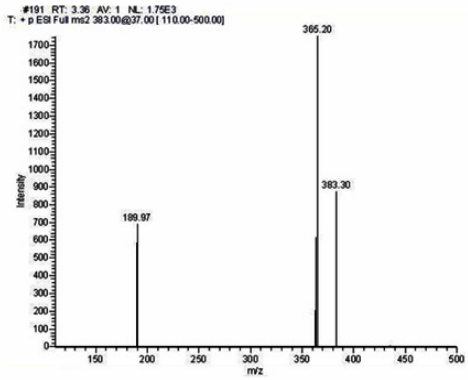


Fig. 7: Fragmentation pattern of the precursor ion *m/z* 383 of the concentrated extract of isolate A11-2.

For comparison, the relative intensities of the produced calcidiol and calcitriol by the five positive isolates (A11-2, A13-4, A8-4, A26-7 and A26-8), as analyzed by MS/MS, were drawn in Fig. 8. As shown in the figure, the isolate A11-2 exhibited the highest product intensity for both calcitriol and calcidiol and it was selected and identified.

Identification of the isolate A11-2.

Microscopical, cultural and biochemical characteristics of the isolate A-11 as compared to those of the standard *Amycolata autotrophica* NRRL B-11275 are shown in Table 1.

Biolog microbial identification system assay

The results of Biolog microbial identification system assay revealed that the test isolate A11-2 was idenitified as *Actinomyces hyovaginalis* since its metabolic fingerprint indicated 100% similarity with *Actinomyces hyovaginalis* when compared with the GP database.

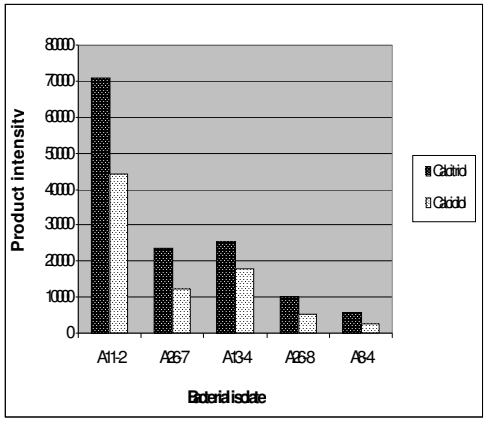


Fig. 8: Relative intensities of the produced calcidiol and calcitriol by the five positive isolates as analyzed by MS/MS. The intensity of each of calcitriol and calcidiol represents the additive intensities of their corresponding precursor ions (*m/z* 416 and 399 for calcitriol and *m/z* 400 and 383 for calcidiol).

Table 1. Microscopical, cultural and biochemical characteristics* of the isolate A11-2 in comparison to standard *Amycolata autotrophica* NRRL B-11275

Test/ Character	***Amycolata autotrophica* NRRL B-11275**	**Isolate A11-2**
Gram stain	Gram positive	Gram negative
Acid fast stain	Non acid-fast	Non acid-fast
Presence of aerial mycelium	present	present
Growth at 5% NaCl	+	+
Mannitol salt agar/ acid	+/+	+/+
production	+/+	
MacConkey agar/ acid	-	+/+
production	+	+
Survival at 50°c for 8 hrs	+	-
Catalase production	+	+
Gelatinase production	+	-
Starch hydrolysis	+	+
Casein hydrolysis	+	+
L-Tyrosine decomposition	+	+
Urease production	+	+
Citrate utilization		
Resistance to ampicillin	+	+
	+	+
Acid production from	+	+
sugars:	+	+
D-Glucose	-	-
Lactose	+	+
Galactose	+	+
Sucrose	+	+
D-Sorbitol	+	-
Maltose		
D(+)Mannose		
Mannitol		
Salicin		

4. Discussion

A screening program was applied on 180 collected isolates for scoring their activity for vitamin D_3 transformation. The concentrated extracts, obtained after biotransformation and extraction processes, were analyzed for all isolates by TLC for calcidiol and calcitriol production. Out of the screened isolates, the five isolates, coded A11-2, A13-4, A8-4, A26-7 and A26-8, were found to be able to convert vitamin D_3 into calcitriol via an intermediate 25-hydroxy vitamin D_3. The concentrated extract of each of these isolates showed TLC spots having the same R_f values as those of 1α-hydroxyvitamin D_3 (0.45) and calcitriol (0.25). Mass spectrometry analyses (MS and MS/MS) were used to confirm the production of calcidiol and calcitriol in the biotransformation mixture and as a quantitative method to compare the biotransformation abilities of the five positive isolates. In the present study, both precursor ions of calcitriol (*m/z* 416 and 399) were detected by MS and MS/MS analyses in the concentrated extracts of each of the five positive isolates confirming their capabilities for biotransformation of vitamin D_3.

The precursor ion *m/z* 416 was referred to the dihydroxylated derivative of vitamin D_3 (calcitriol) (*m/z* 384 (for vitamin D_3) +32 (for two hydroxyl groups) as reported by Holick *et al*. (1971) and Ying *et al*. (2009). Also, another precursor ion *m/z* 399 was referred to calcitriol and this ion is formed by loss of the hydroxyl group at carbon number 3 (Capote *et al*., 2007;Ying *et al*., 2009).

Concerning the intermediate; calcidiol, two precursor ions (*m/z* 400 and 383) were detected by MS and MS/MS analyses in the concentrated extracts of each of the five positive isolates confirming the formation of calcidiol as an intermediate during biotransformation of vitamin D_3 into calcitriol by the five positive isolates. The precursor ion *m/z* 400 was referred to the monohydroxylated derivative of vitamin D_3 (calcidiol) (*m/z* 384 (for vitamin D_3) +16 (for one hydroxyl group), as reported by Ying *et al*. (2009). Also, another precursor ion *m/z* 383 was referred to calcidiol and this ion is formed by loss of the hydroxyl group at carbon number 3 (Capote *et al*., 2007;Ying *et al*., 2009).

Further confirmation of the production of calcidiol and calcitriol, by the five positive isolates capable of biotransforming vitamin D_3, was carried out by fragmentation of their precursor ions detected by MS/MS at high collision energy. For the precursor ion *m/z* 416, which was detected in both calcitriol standard preparation and the concentrated extracts of the five positive isolates (test preparations), the fragmentation resulted in the appearance of a characteristic fragment ion *m/z* 343 in both standard and test preparations. Such finding gave a clear evidence for calcitriol as a biotransformation product in the concentrated extracts of the five positive isolates. Upon using a software program (ACD/ChemSketch, Advanced Chemistry Development, Inc., Toronto, Canada), commonly used by several authors for structural elucidation of different fragment ions, for prediction of fragmentation pattern of calcitriol, it was found that a fragment ion *m/z* 343 is among the different fragment ions of calcitriol. The suggested structure of this fragment ion produced by the software program is shown in Fig. 9.

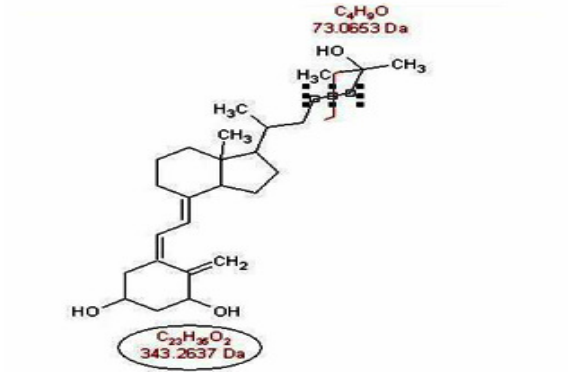


Fig. 9: Suggested structure of fragment ion *m/z* 343 produced by fragmentation of calcitriol as predicted by ACD/ChemSketch program.

For the precursor ion *m/z* 399, which was detected only in the test preparations, the fragmentation resulted in the appearance of a characteristic fragment ion *m/z* 381 in all cases together with non fragmented precursor ion *m/z* 399 at low intensity and other non characteristic fragment ions. A similar finding was reported by Capote *et al*. (2007) and Ying *et al*. (2009). They found the fragment ion *m/z* 381 during fragmentation of calcitriol precursor ion *m/z* 399. The structure of the fragment ion suggested by the authors is shown in Fig. 10.

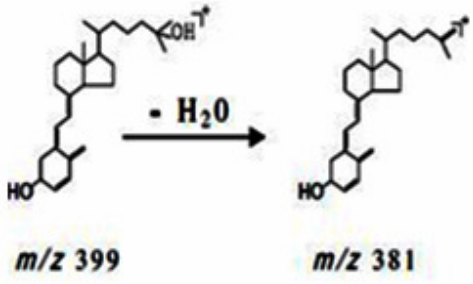


Fig. 10: Suggested fragmentation pattern of calcitriol precursor ion m/z 399 producing fragment ion m/z 381 (Capote *et al*., 2007; Ying *et al*., 2009).

Concerning calcidiol and for the precursor ion *m/z* 400, which was detected in the test preparations, no fragment ions appeared upon raising the collision

energy. Whereas, for the precursor ions *m/z* 383, which was detected in the test preparations, the fragmentation resulted in the appearance of a characteristic fragment ion *m/z* 365 in all cases together with non fragmented precursor ion *m/z* 383 at low intensity and other non characteristic fragment ion. A similar finding was reported by Capote *et al*. (2007) and Ying *et al*. (2009). They found the fragment ion *m/z* 365 during fragmentation of calcidiol precursor ion *m/z* 383. The structure of the fragment ion suggested by the authors is shown in Fig. 11.

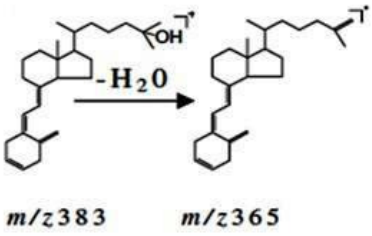


Fig. 11: Suggested fragmentation pattern of calcidiol precursor ion *m/z* 383 producing fragment ion *m/z* 365 (Capote *et al*., 2007; Ying *et al*., 2009).

The results of MS, MS/MS and fragmentation patterns of the detected precursor ions gave a convincing evidence for the presence of both calcidiol and calcitriol as biotransformation products of vitamin D_3 by the five positive isolates. Moreover, the isolate A11-2 exhibited the highest product intensity for both calcitriol and calcidiol, as determined by MS/MS analysis, and this isolate was selected and identified.

Paraffin baiting based isolation and microscopical criteria of isolate A11-2, preliminarily, suggested that it belonged to *Nocardia*/*Amycolata* or *Actinomyces* genus. Identification results obtained showed great similarity between the test isolate A11-2 and the standard strain, *A. autotrophica* NRRL B-11275. They only differed from each other in that isolate A11-2 could survive at 50°C for 8 hrs, showed weak growth at 50°C after incubation for 7 days and could not produce gelatinase enzyme. Moreover, isolate A11-2 could not produce acid from salicin while *A. autotrophica* NRRL B-11275 could produce. By conducting the Biolog microbial identification system assay, the resulting metabolic fingerprint indicated 100% similarity with *Actinomyces hyovaginalis* when compared with the GP database. Accordingly, the test isolate A11-2 is a member of such bacterial species.

Interestingly and to the best of our knowledge, the results obtained in the present study reveal, for the first time, that vitamin D_3 can be transfomed into calcidiol and calcitriol by *Actinomyces* species. Consequently, our test isolate *Actinomyces hyovaginalis* can be considered as a prototype for biotransformation of vitamin D_3 into its fully active metabolite; calcitriol among members of *Actinomyces* species.

Coresponding author
Mohammad M. Aboulwafa
Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, Organization of African Unity St., POB: 11566, Abbassia, Cairo, Egypt
maboulwafa@yahoo.com

References

Bligh EG, Dyer WJ (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911.

Bouillon RW, Okamura H, Norman HAW (1995). Structure-function relationships in the vitamin D endocrine System. Endocrine Rev., 16: 200-257.

Capote FP, Jimenez JR, Granados JMM, De Castro MDL (2007). Identification and determination of fat-soluble vitamins and metabolites in human serum by liquid chromatography/triple quadrupole mass spectrometry with multiple reaction monitoring. Rapid Commun. Mass Spectrom, 21: 1745–1754.

Collins CH, Lyne PM, Grange JM, Falkinham JO (2004). Identification methods. In: Collins CH, Lyne PM, Grange JM, Falkinham JO. Collins and Lyne's Microbiological methods, 8th ed. Edward Arnold, London. pp: 89-109.

DeLuca HF, Schnoes HK (1983). Vitamin D: recent advances. Annu. Rev. Biochem., 52: 411–439.

DeLuca HF (2004). Overview of general physiologic features and functions of vitamin D. Am. J. Clin. Nutr., 80: 1689S-1696S.

Eastell R, Riggs BL (1997). Vitamin D and osteoporosis. In: Feldman D, Glorieux FH, Pike JW Vitamin D. Academic Press, New York. pp. 695–711.

Foster PR, Dunnill P, Lilly MD (1971). Salting-Out of Enzymes with Ammonium Sulphate. Biotech and Bioeng.,13: 713-718.

Gordon RE, Barnett DA, Handerhan JE, Pang CHN(1974). *Nocardia coeliaca, Nocardia autotrophica*, and the nocardin strain. Int. J. Syst. Bacteriol., 24: 54–63.

Gordon RE, Hagan WA (1936). A study of acid fast *Actinomycetes* from soil with special reference to pathogenicity for animals. J. Infect. Dis., 59: 200-206.

Grant WB, Holick MF (2005). Benefits and requirements of vitamin D for optimal health: a review. Altern. Med. Rev., 10 : 94–111.

Holick MF, Schnoes HK, Deluca HF (1971). Identification of 1, 25-dihydroxycholecalciferol, a

form of vitamin D_3 metabolically active in the intestine. Proc. Nat. Acad. Sci., 68: 803-804.

Holt JG (1994). Groups with the four major categories of bacteria. In: J. G. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Staley and S. T. Williams. Bergey's manual of determinative bacteriology, 9th ed. The Williams & Wilkins Co., Baltimore. pp. 605-650.

Kametani T, Furuyama H(1987). Synthesis of vitamin D_3 and related compounds, Med. Res. Rev., 7: 147–171.

Koser SA (1924). Correlation of citrate utilization by members of the colon-aerogenes groups with other differential characteristics and with habitat. J. Bacteriol. , 9: 59-77.

Larry EJ (2007). "Vitamin D". The Merck manuals. http://www.merckmanuals.com/professional/sec01/ch004/ch004k. [accessed 12/11/2009].

Levine M, Carpenter DC (1923). Gelatin liquefaction by bacteria. J. Bacteriol. , 8: 297.

McCollum EV, Simmonds N, Parsons HT, Shipley PG, Park EA (1921). Studies on experimental rickets. I. The production of rachitis and similar diseases in the rat by deficient diets. J. Biol. Chem. , 45: 333 – 342.

Miller JH (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. pp: 433.

Rowe B, Gross RJ (1984). Genus II *Shigella*. In: Krieg NR and Holt JG. Bergey's Manual of Systematic Bacteriology, 1st ed., vol. 1. The Williams and Wilkins Co., Baltimore. pp. 423-427.

Rustigian R, Stuart CA1941). Decomposition of urea by *Proteus*. Proc. Soc. Exp. Biol. Med, 47: 108.

Sasaki J, Mikami A, Mizoue K, Omura S (1991). Transformation of 25- and 1α-hydroxyvitamin D_3 to 1α, 25-dihydroxyvitamin D_3 by using *Streptomyces* sp. strains. Appl. Environ. Microbiol. , 57: 2841-2846.

Sasaki JA, Miyazaki M, Saito T, Adachi K, Mizoue K. Hanada, Omura S (1992). Transformation of vitamin D_3 to lα, 25-dihydroxyvitamin D_3 via 25-hydroxyvitamin D_3 using *Amycolata* sp. strains. Appl. Microbiol. Biotechnol. , 38: 152–157.

Seino Y, Tanaka H, Yamaoka K, Yabuuchi H (1987). Circulating 1α, 25-dihydroxyvitamin D levels after a single dose of 1 α, 25-dihydroxyvitamin D_3 or 1 α -hydroxyvitamin D_3 in normal men. Bone Miner. , 2: 479–485.

Vedder EB (1915). Starch agar, a useful culture medium. J. Infect. Dis. 16: 385.

Ying Y, Yingyu P, Yujuan C, Jiazhong W, Guxian L (2009). Identification and structural elucidation of vitamin D_3 metabolites in human urine using LC-MS-MS. Chromatographia. , 69: 103-109.