SYNTHESIS OF LABELLED ECDYSONE PRECURSORS : PART I – TRITIUM LABELLED ($^3H_4$-22,23,24,25)-3β,14α-DIHYDROXY-5β-CHOLEST-7-EN-6-ONE.

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SUMMARY.

High specific activity tritiated 3β,14α-dihydroxy-5β-cholest-7-en-6-one, has been prepared using a precursor which permits rapid and easy labelling. This compound is converted to ecdysone under *in vitro* conditions by insect prothoracic glands, a well known site of ecdysone biosynthesis.

Key words : Ecdysone precursor, biosynthesis, insects, prothoracic glands .

INTRODUCTION

The essential events of development and reproduction in arthropods, and probably also in other invertebrate classes, are controlled by steroid

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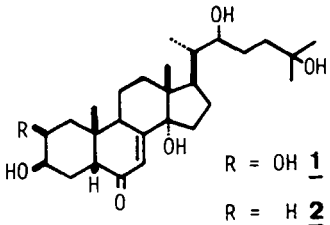
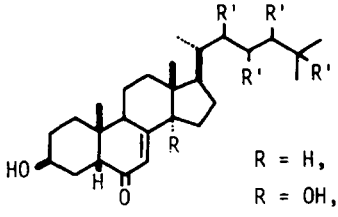
hormones, called ecdysteroids ("molting hormones"). The biosynthetic pathway(s) leading to ecdysone 1 has been the object of a few investigations in a limited number of biological models (review in 1). Practically no data are available on the enzyme systems which are involved in ecdysone biosynthesis.



R = OH 1

R = H 2



R = H, R'= H 3

R = OH, R'= H 4

R = OH, R'= ^{3}H 4'

In a previous study on the biosynthetic pathway of ecdysteroids in ovaries of Locusta we proposed the following preferential sequence of events leading to ecdysone 1 from cholesterol : Conversion of cholesterol to 3β-hydroxy-5β-cholest-7-en-6-one 3, followed by successive hydroxylations at C-14, C-25, C-22 and C-2.

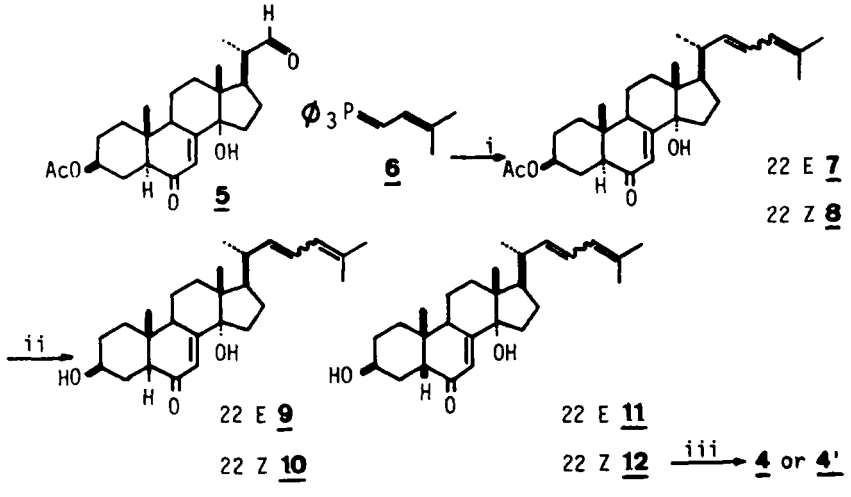
The unequivocal demonstration of this pathway in ovaries, its extension to other invertebrate systems and the study of the enzymes involved in ecdysone biosynthesis required the utilisation of high specific activity labelled precusor molecules. We have recently published the synthesis of ($^{3}H_4$-23,24)-2-deoxyecdysone 2 and we now report a rapid and facile synthesis of ($^{3}H_4$-22,23,24,25)-2,22,25-trideoxyecdysone 4' (3β,14α-dihydroxy-5β-cholest--7-en-6-one or 5β-ketodiol 4) with high specific activity. In addition, we have ascertained that this molecule is actually converted to ecdysone 1 under in vitro conditions by intact and homogenized prothoracic glands.

Although compound 4' has already been synthesized (2), the route used was not appropriate for radioactive labelling.

The synthetic pathway which we now propose enables the production of appreciable amounts of radiolabelled 5β-ketodiol **4'** with the tritium labels at several positions on the side chain : C-22, C-23, C-24 and C-25. During the conversion of this molecule to ecdysone **1**, almost all of the label was retained, which makes this compound a useful tool for biosynthetic studies.

RESULTS

The ylide **6** was prepared in hexane from a suspension of the bromide salt (3, 4), by addition of n-butyl lithium, and was then condensed with the aldehyde **5**, the synthesis of which has been described elsewhere (5). We obtained a mixture of C-22 olefinic isomers, **7** and **8**, (with a ratio of Z/E : 60/40).



i : BuLi, Hexane; ii : K_2CO_3, MeOH; iii : Pd/C 5%, AcOEt.

Attempts to separate these isomers using $AgNO_3$ loaded SiO_2 were unsuccessful (4). The mixture of **7** and **8**, was deacetylated and epimerized at C-5 to give a mixture of four isomers. These were then separated by high-performance liquid chromatography on a reversed phase column. Isomers **9**, **10**, **11** and **12** were obtained in the relative proportion of 31, 46, 10 and 13.

In the ecdysteroid family, only the 5β-H isomer is biologically active (6) and in our biosynthetic scheme the 5β form appears immediately after the introduction of the unsaturated ketone at C-6. In steroids containing a 2β-hydroxyl group the formation of the 5β-epimers (A/B cis junction) is favoured by a steric 1,3 diaxial interaction between the 2β-hydroxyl and the C-19 methyl group (7, 8). But in the ketodiol **4** where this steric interaction is absent, the 5α-H epimer was expected to be more stable which proved to be true with the resulting ratio of 5α-H isomer over 5β-H isomer being 4.

Finally hydrogenation of **12** (the predominant 5β isomer) with 5% Pd/C gave quantitatively the ketodiol **4**. Tritiation performed at the Commissariat à l'Energie Atomique (CEN, Saclay, France) yielded ^{3}H-ketodiol **4'** with a specific activity of 107 Ci/mmole.

BIOLOGICAL ACTIVITY.

Prothoracic glands were excised from three larvae of *Locusta* of the mid fifth instar (period of intense ecdysone **1** synthesis *in vivo*) and incubated in Landureau's medium (personnal communication) in the presence of $0.5\ 10^{-7}$ M of the newly-synthesized radiolabelled 5β-ketodiol **4'**. After 4 h, the glands (plus the medium) were homogenized in a glass teflon potter in 50% aqueous ethanol. The homogenate was heated to 60°C for 15 min and centrifuged at 800 g for 5 min. The pellet was reextracted twice with 95% ethanol and the supernatants were combined and reduced in volume under a stream of nitrogen. The labelled compounds were then separated by silica gel thin-layer chromatography in chloroform-methanol (80v/20v), and several radioactivity

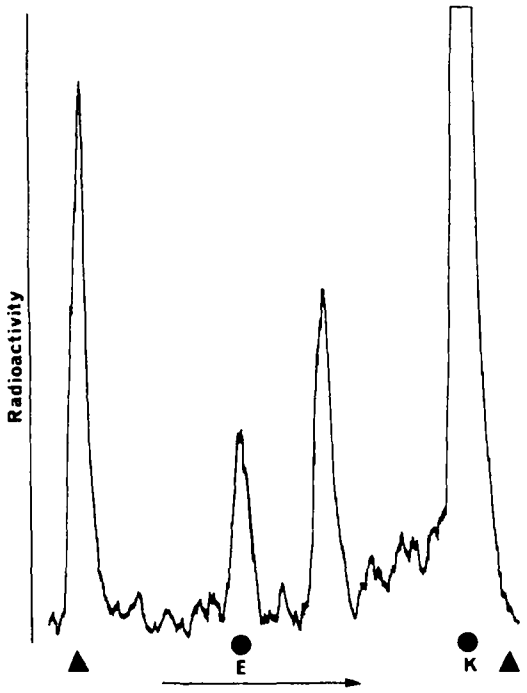


Figure 1 : Radiochromatogramme of the metabolites of the 5β-ketodiol **4'** in the incubation medium and in the prothoracic glands after a 4 h incubation. The ethanolic extract of the homogenate was subjected to a silicagel thin layer chromatographical separation (solvent system, chloroform : ethanol, 4v: 1v). Arrow heads, start and front of migration; arrow, sense of migration; E, K, migration reference ecdysone and 5β-ketodiol.

peaks were monitored one of which comigrated with authentic ecdysone. (Fig 1). The molecules of this peak were eluted with 95% ethanol and subjected to reverse phase HPLC (Bondapak, 55% aqueous methanol, isocratic elution) where the radioactivity co-eluted with authentic ecdysone (Fig 2). The final identification of the radiolabelled ecdysone formed *in vitro* from tritiated 5β-ketodiol **4'** was based on co-acetylation with authentic ecdysone.

In these experiments approximatively 1.7% of the labelled 5β-ketodiol **4'** was converted to ecdysone **1**, 87% of the ketodiol remained unchanged, the rest of the radioactivity was distributed among several as yet unidentified radioactivity peaks.

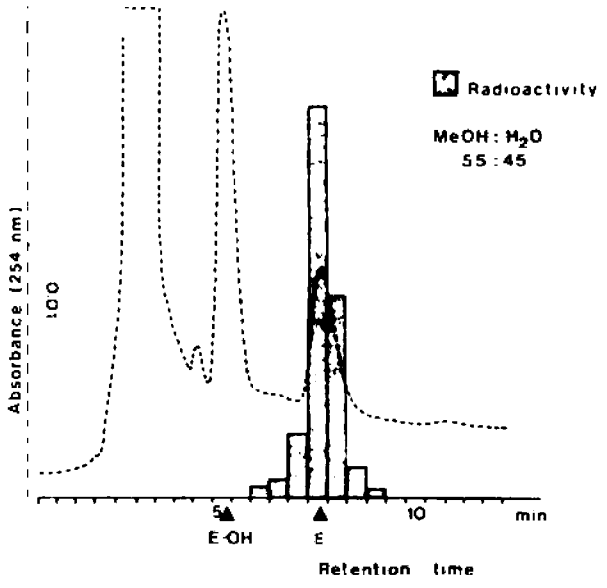


Figure 2 : HPLC analysis of the fraction co-migrating with reference ecdysone on a silcagel plate (Fig. 1) : the eluted labelled metabolite was subjected to a C-18 reverse phase separation; isocratic elution in methanol:water (55:45). Dotted line, U.V. absorbance; colums, radioactivity measurements of aliquots of each fraction. E-OH, E, elution of reference 20-hydroxyecdysone and ecdysone.

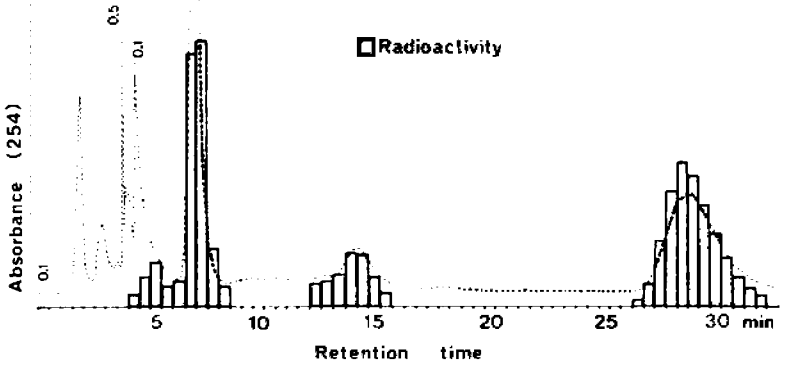


Figure 3 : Co-acetylation of putative labelled ecdysone synthesized by the prothoracic glands in an acellular system and reference unlabelled ecdysone; C-18 reverse phase column; isocratic elution in methanol-water (65:35). Dotted line, U.V. absorbance; columns, radioactivity measurements of aliquots of each fraction.

The yield of conversion was noticeably increased when the prothoracic glands of twenty larvae of Locusta were homogenized (200 mM phosphate buffer, pH 7.2) and the 800 g supernatant incubated with 10^{-7}M tritiated 5β-ketodiol 4' (same buffer, supplemented with 200 µ M glucose-6-phosphate, 200 µ M glucose-6-phosphate dehydrogenase and 200 µ M NADPH). Up to 30% of the label was recovered after 4 h in the ecdysone peak. The identity of ecdysone was ascertained by co-acetylation with reference (Fig 3) of the labelled putative ecdysone purified by thin-layer chromatography and isocratic HPLC.

CONCLUSION

Tritiated 5β-ketodiol obtained with high specific activity should now allow investigations on the later steps of ecdysone biosynthesis in a variety of biological models and on the enzyme systems involved and their regulation during development.

EXPERIMENTAL

Melting point were measured on a microscopic Reichert apparatus and are uncorrected, $[\alpha]_D$ were measured on a Perkin Elmer 141 polarimeter, IR spectra were recorded in KBr on a Perkin Elmer 177, UV spectra were measured on a Jobin Yvon Duospac 203 UV-Vis spectrometer, NMR were recorded on a Bruker SY (200 MHz) apparatus, in deuteriochloroform or in deuteriomethanol, and TMS as internal standard, MS were measured on a LKB 9000 S apparatus by direct introduction without derivatization, HPLC was carried on a Du Pont intrument incorporating a 870 Pump module with a model 8800 Gradient controller.

3β-Acetoxy-14α-hydroxy-5α-cholesta-7,22 E,24-trien-6-one (7) and 3β-acetoxy-14α-hydroxy-5α-cholesta-7,22 Z,24-trien-6-one (8).

The ylide 6 was prepared by addition of 11 mmoles of n butyl lithium in a suspension of 6.8g (16.6 mmoles) of the phosphonium bromide salt, at 20°C, with vigourous stirring. After 1 h , a solution of 1.06 g (2.63 mmoles) of 5

was added dropwise at room temperature. The resulting reaction mixture was then stirred for 1h at the same temperature. The suspension was then cooled, filtered and the precipitate washed with cold dichloromethane. The filtrate was chromatographed on SiO_2 (AcOEt/Hexane as eluant) to yield 695 mg of the mixture **7** and **8** (58%). 150 mg of the mixture was separated by low pressure chromatography on SiO_2 with Hexane/AcOEt, 75/25, as eluant. However, though the ratio of **8**/**7** was 2/3 (deduced from the U.V. detection spectrum) we only recovered 20 mg of pure **7** and 80 mg of pure **8**.

3β-Acetoxy-14α-hydroxy-5α-cholesta-7,22 E,24-trien-6-one (**7**).

m.p. : 197-198 C°

I.R. cm-1 : 3600-3200, 1730, 1650, 1625, 1470, 1435, 1380, 1240, 1035.

^1H NMR in Table I and ^{13}C NMR in Table II

M.S. : m/z 454 (74%)(M+, $C_{29}H_{42}O_4$) 436 (10%), 373 (100%), 313 (45%), 256 (11%), 109 (15%), 82 (45%), 60 (14%).

$[\alpha]_D$ = 27° (c = 5, $CHCl_3$).

UV λ_{max} = 238 nm, ε = 34000.

3β-acetoxy-14α-hydroxy-5α-cholesta-7,22 Z,24-trien-6-one (**8**).

m.p. : 199-200 C°

I.R. cm-1 : 3600-3200, 1730, 1650, 1625, 1450, 1380, 1365, 1240, 1035.

^1H NMR in Table I and ^{13}C NMR in Table II

M.S. : m/z 454 (36%)(M+, $C_{29}H_{42}O_4$) 436 (28%), 373 (100%), 313 (74%), 256 (32%), 109 (42%), 82 (84%), 60 (82%).

$[\alpha]_D$ = 7° (c = 13, $CHCl_3$).

UV λ_{max} = 239 nm, ε = 31000.

3β,14α-Dihydroxy-5α-cholesta-7,22 E,24-trien-6-one (9), 3β,14α--dihydroxy-5α-cholesta-7,22 Z,24-trien-6-one (10), 3β,14α-dihydroxy-5β--cholesta-7,22 E,24-trien-6-one (11) and 3β,14α-dihydroxy-5β-cholesta--7,22 Z,24-trien-6-one (12).

The unseparated mixture of **7** and **8** (200 mg, 0.5 mmoles) in 25 ml MeOH were heated under reflux and then treated with a solution of 138 mg (1 mmoles) of K_2CO_3 in 3 ml of H_2O. After 10 min the reaction was cooled to -10°C, neutralised with 1 N HCl and extracted with AcOEt. The mixture of 4 isomers **9**, **10**, **11** and **12** was purified by HPLC on a preparative ODS column (Zorbax, ODS, 21.1x250mm, 5 μm) with MeOH/H_2O (80/20), flow rate 10 ml/min. 147 mg (65%) of separated isomers was obtained. The relative quantity of each is **9** : 31%, **10** : 46%, **11** : 10%, **12** : 13%.

Table I ^{1}H NMR signals (in $CDCl_3$ (1) or in CD_3OD (2), ppm from TMS).

For all compounds :

(a = axial, e = equatorial referring to the A-cycle for 3-H and 5-H, and to the C-cycle for 9-H ; d= doublet ; m= multiplet)

5-H: X part of an ABX system with $\nu_{4a}-\nu_{4b} \simeq 15$ Hz, J_{5a-4a}(apparent) $\simeq$ 12.5 Hz J_{5a-4e}(apparent) $\simeq$ 3.7 Hz

7-H: d, $J \simeq 2.7$ Hz; 21-H: d, $J \simeq 6.5$ Hz;

9-H: doublet of the X- part of an ABX system with $\nu_{11a}-\nu_{11e} \simeq 5$ Hz $J_{9a-6} \simeq 3$ Hz, J_{9a-11e}(apparent) $\simeq$ 7 Hz, J_{9a-11a}(apparent) $\simeq$ 11 Hz

E isomers: $J_{22-23} \simeq 15$ Hz; $J_{23-24} \simeq 11$ Hz; $J_{22-20} \simeq 8.5$ Hz;

Z isomers: ABX system (22-H, 23-H, 24-H) with $\nu_{24-H}-\nu_{23-H} \simeq 5$ Hz; J_{AB}(apparent) $\simeq$ 8.5 Hz;22-H: m, $w_{1/2} \simeq 20$ Hz;

5α isomers: 3-H(a): m, $w_{1/2} \simeq 25$ Hz;

5β isomers: 3-H(e): m, $w_{1/2} \simeq 13$ Hz.

Mol.	5(1)	7(1)	8(1)	9(2)	10(2)	11(2)	12(2)	4(2)
H-N°								
3-H	4.69	4.72	4.71	3.57	3.58	4.01	4.01	4.01
5-H	2.37	2.34	2.33	2.38	2.38	2.44	2.44	2.45
7-H	5.91	5.90	5.89	5.87	5.85	5.82	5.82	5.83
9-H	2.74	2.73	2.73	2.79	2.75	3.22	3.22	3.23
18-H	0.72	0.70	0.74	0.76	0.80	0.76	0.80	0.74
19-H	0.88	0.88	0.88	0.89	0.90	1.00	1.00	1.00
21-H	1.15	1.06	1.01	1.08	1.03	1.08	1.03	0.95
22-H	9.59	5.42dd	5.15m	5.41dd	5.17m	5.42dd	5.17m	-
23-H	-	6.19dd	6.08	6.20dd	6.12	6.22dd	6.12	-
24-H	-	5.76d	6.04	5.75d	6.07	5.76d	6.08	-
26-H	-	1.74	1.75	1.76	1.77	1.76	1.77	0.91
27-H	-	1.76	1.81	1.76	1.83	1.76	1.83	0.94

Table II ^{13}C NMR signals (in $CDCl_3$ (1) or in CD_3OD (2), ppm from TMS).

Subst.	5(1)	7(1)	8(1)	9(2)	10(2)	11(2)	12(2)	4(2)
C N°								
3	72.8	72.7	72.8	72.1	72.1	66.4	66.4	66.4
5	53.4	53.2	53.3	55.5	55.5	53.2	53.2	53.2
6	199.7	199.4	199.8	204.0	204.0	207.3	207.3	207.4
7	123.0	122.7	122.7	124.2	123.3	122.7	124.3	122.6
8	170.6	162.8	163.2	167.3	167.3	169.2	169.2	169.3
11	20.5	20.4	20.6	22.6	22.6	23.0	23.0	23.0
14	84.1	84.9	85.1	86.3	86.3	86.8	86.7	86.8
17	46.1	50.3	50.7	52.7	52.9	52.7	52.9	52.8
18	16.3	16.0	16.3	17.5	17.7	17.5	17.7	17.2
19	13.7	12.8	13.0	14.2	14.2	25.3	25.3	25.3
21	13.0	12.8	13.0	19.1	19.0	19.1	18.9	20.3
22	204.4	137.5	135.5	139.9	137.7	139.9	137.6	-
23		124.7	120.5	127.5	122.6	127.4	122.6	-
24		125.0	122.4	126.5	122.6	126.9	122.6	-
25		132.8	135.1	134.1	136.5	134.1	136.5	30.0
26		-	-	22.4	22.3	22.4	22.3	23.8
27		-	-	-	-	-	-	24.4

3β,14α-Dihydroxy-5α-cholesta-7,22 E,24-trien-6-one (**9**).

m.p. : 199-201 °C

I.R. cm^{-1} : 3600-3200, 1660, 1440, 1380, 1055.

^{1}H NMR in Table I and ^{13}C NMR in Table II.

M.S. : m/z 412 (17%)(M+, $C_{27}H_{40}O_3$) 394 (10%), 379 (4%), 331(100%), 313 (10%), 302 (3%), 285 (13%), 234 (6%), 109 (60%), 82 (45%).

$[\alpha]_D$ = 32° (c = 8.3, MeOH).

UV λ_{max} = 238 nm, ε = 33000.

3β,14α-dihydroxy-5α-cholesta-7,22 Z,24-trien-6-one (**10**).

m.p. : 211-213 °C

I.R. cm^{-1} : 3600-3200, 1645, 1450, 1385, 1075, 1050, 1035.

^{1}H NMR in Table I and ^{13}C NMR in Table II.

M.S. : m/z 412 (17%)(M+,$C_{27}H_{40}O_3$) 394 (6%), 379 (4%), 331 (100%), 313 (13%), 302 (3%), 285 (13%), 234 (6%), 109 (90%), 82 (95%).

$[\alpha]_D$ = -5° (c = 12.2, MeOH).

UV λ_{max} = 239 nm, ε = 26000

3β,14α-dihydroxy-5β-cholesta-7,22 E,24-trien-6-one (**11**).

I.R. cm-1 : 3600-3200, 1650, 1440, 1380, 1040.

1H NMR in Table I ^{13}C NMR in Table II

M.S. : m/z 412 (13%)(M+, $C_{27}H_{40}O_3$) 394 (4%), 383 (0.5%), 379 (2%), 331 (91%), 313 (5%), 302 (5%), 285 (11%), 234 (8%), 109 (100%), 82 (87%).

$[\alpha]_D$ = 66° (c =5.5, MeOH).

UV λ_{max} = 238 nm, ε = 33000

3β,14α-dihydroxy-5β-cholesta-7,22 Z,24-trien-6-one (**12**).

m.p. : 204-206 °C

^{1}H NMR in Table I and ^{13}C NMR in Table II.

M.S. : m/z 412 (10%)(M+,$C_{27}H_{40}O_3$) 394 (4%), 383 (1%), 379 (2%), 331 (100%), 313 (6%), 302 (4%), 285 (10%), 234 (9%), 109 (81%), 82 (63%).

$[\alpha]_D$ = 31° (c = 2.7, MeOH)

UV λ_{max} = 239 nm, ε = 31000.

3β,14α-dihydroxy-5β-cholest-7-en-6-one. (4).

The ketone **12** (13 mg, 0.03 mmole) in 2ml AcOEt was hydrogenated in the presence of 8 mg of Pd/C 5% for 1 h at room temperature. After filtration of the catalyst, we obtain **4** quantitatively.

3β,14α-dihydroxy-5β-cholest-7-en-6-one (4).

m.p. : 172-173°C

I.R. cm^{-1} : 3600-3200, 1650, 1450, 1380, 1035.

^{1}H NMR in Table I and ^{13}C NMR in Table II.

$[\alpha]D$ = 80.4° (c = 5.6, MeOH).

U.V. λ_{max} = 245 nm, ϵ = 12000.

M.S. :m/z 416 (30%)(M^+, $C_{27}H_{44}O_3$) 398 (12%), 388 (100%), 383 (15%), 355 (20%), 313 (6%), 302 (3%), 285 (7%), 233 (68%).

High-Resolution-Mass-Spectrometry : $M^+(C_{27}H_{44}O_3)$

found : 416.3285 0.002 , calc. : 416.3290

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