STRUCTURAL STUDIES ON MINOR COMPONENTS OF SAFRAMYCIN GROUP ANTIBIOTICS SAFRAMYCINS F, G AND H

YUZURU MIKAMI, KATSUHIRO TAKAHASHI, KATSUKIYO YAZAWA, CHEN HOUR-YOUNG and TADASHI ARAI

Department of Experimental Chemotherapy, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1 Inohana, Chiba, Japan

NAOKI SAITO and AKINORI KUBO

Department of Organic Chemistry, Meiji College of Pharmacy, 1-35-23 Nozawa, Setagaya-ku, Tokyo, Japan

(Received for publication January 25, 1988)

Three minor components of saframycin group antibiotics, saframycins F, G and H were isolated and their structures were determined by comparison with the spectral data of UV, IR, and ^{1}H and ^{13}C NMR of already reported saframycins A and D. Saframycins F, G and H were 21-cyanosaframycin D, 14-hydroxysaframycin A and 25-dihydro-25-β-ketopropyl-saframycin A, respectively.

Structures of 13 saframycin components, saframycins A, B, C, D, S, Y3, Yd-1, Yd-2, Ad-1, AR1, AR3, Y2b and Y2b-d including recently prepared dimeric products by directed biosynthesis method[1], have been determined. However, due to insufficient materials, structures of three minor components *i.e.*, saframycins F, G and H have not yet been established. In this paper, isolation, physico-chemical characterization and structural elucidations of these minor components are described.

Isolation of Saframycins G, F and H

In our previous papers[2~4], we reported that pH control of culture after the logarithmic growth phase and sodium cyanide treatment of culture filtrate increased saframycin A production. The treatments were also found to increase concomitantly the amount of other minor components of saframycin group antibiotics.

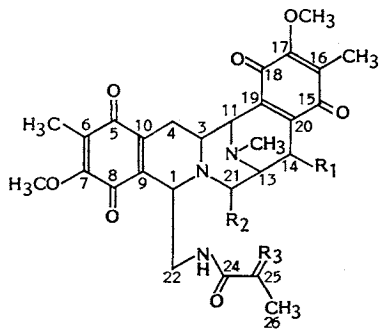
Detailed purification procedures are described in the Experimental section.

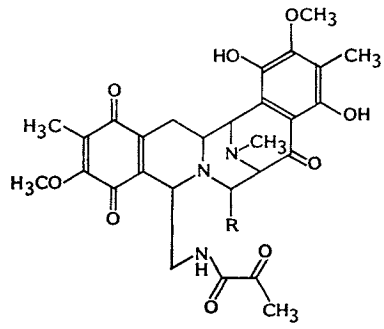
Structure of Saframycins

Saframycin F (SF)

SF is a weakly basic, faint yellow powder which decomposes at 134~136°C. The UV spectrum is different from that of saframycin A (SA), a main component of the saframycin group of antibiotics and bears a somewhat greater similarity to saframycin D (SD). On the basis of elemental analysis and mass spectral data (*m/z* 578), the molecular formula was determined as $C_{29}H_{30}N_4O_9$. This formula shows that SA has one carbon and one nitrogen atoms more than and one hydrogen atom less than SD. The ^{13}C NMR spectrum of SF is compared with that of SA and SD in Table 2. The spectral data of SF and SD showed a good agreement. These data indicate that the basic carbon skeleton of SF is identical or very similar to that of SD; therefore, further structural studies on SF were done

Fig. 1. Structures of saframycins.





Saframycin A R_1 = H R_2 = CN R_3 = O
Saframycin B R_1 = R_2 = H R_3 = O
Saframycin C R_1 = OCH_3 R_2 = H R_3 = O
Saframycin G R_1 = OH R_2 = CN R_3 = O
Saframycin H R_1 = H R_2 = CN R_3 = OH, CH_2COCH_3

Saframycin D R = H
Saframycin F R = CN

in comparison with SD. The appearance of a new signal at 117.1 (s) in SF suggests the presence of a cyanide group in the molecules. The signal at 54.8 (t, C-21) in SD, is not observed in SF. It was further found that all signals of SF in these regions were observed as doublets. The hydrogen atom attached at C-21 is thus thought to be replaced by CN in the SF molecule, and the structure of SF is presented as 21-cyanosaframycin D.

When the ^1H NMR spectra of SF and SD were compared, the signals at 2.93 (21-Hb) and 3.28 (21-Ha) were not observed in SF, but a new signal appeared at 4.26 (1H, d, J=2.9 Hz). These data also supported the above hypothesis that SF is 21-cyanosaframycin D.

In addition to the ion peaks of m/z 553 (M^+), 453 (M^+-100), 319, 236 (base) and 218 observed in SD, new peaks of m/z 578 (M^+), 478 (M^+-100), 344, 243 and 236 (base) were observed in SF. These mass spectral data also indicate that the site of C-21 is substituted by the CN group in SF. The final structure of SF was therefore determined to be 21-cyanosaframycin D (Fig. 1).

Saframycin G (SG)

SG is a basic, amorphous yellow powder which decomposes at 134~136°C. SG gave a major ion peak at m/z 578 (M^+) in an electron impact (EI)-MS. The molecular formula was determined as $C_{29}H_{30}N_4O_9$ based on elemental analysis, indicating that SG possesses one oxygen atom more than SA. The UV and IR spectra also show similarity to SA. However, in contrast to SA, SG revealed a monoacetate derivative (mp 160°C (dec), m/z 620, ^1H NMR 2.08 (3H, s), 5.40 (1H, s)) by the treatment with acetic anhydride - pyridine. This suggests the presence of a secondary hydroxyl group in the SG molecule, and the one increased oxygen atom was considered due to the hydroxyl group. ^1H NMR spectrum of SG also shows good agreement with that of SA, except for minor differences (Table 1). Two signals at 2.83 (14-Ha) and 2.24 (14-Hb) disappeared and a new signal at 4.32 (1H, s) appeared in the spectrum of SG when compared with SA (Table 1). In addition, the signal at 21.6 (t, C-14) disappeared and a new signal of 62.1 (d) appeared in SG. The newly observed signal's chemical shifts were very similar to that of C-14 (71.9, d) and 10-H (3.79, s) in saframycin C (SC). Furthermore, a new characteristic fragment, m/z 234 (218+16), was observed in the EI-MS of SG in addition to the commonly

Table 1. ^{1}H NMR spectral data of newly prepared saframycins in comparison with those of saframycins A and D.

Proton No.	Saframycins									
	A		D		F		G		H	
	δ	J	δ	J	δ	J	δ	J	δ	J
NH	6.86	8.8	6.28	9.0	6.12	9.5	6.63	9.5	6.75	10.3
		4.5		4.0		3.8		4.4		3.4
22-Ha	3.84	8.8	3.71	9.0	3.71	9.5	3.69	9.5	3.84	10.3
		14.3		14.0		14.6		14.3		13.9
		1.5		1.5		1.5		1.6		1.6
22-Hb	3.27	4.5	3.06	4.0	3.05	3.8	3.33	4.4	2.95	3.4
		14.3		14.0		14.6		14.3		13.9
		4.5		3.0		3.8		4.4		3.4
1-H	3.98	1.5	3.68	4.0	3.98	(br s)	3.96	(br s)	4.02	(br s)
		4.5		3.0						
		2.9		1.5						
4-Hb	1.29	2.9	1.58	3.0	1.53	2.6	1.21	3.2	1.43	3.2
		17.6		18.0		17.8		17.3		17.3
		11.7		11.0		11.3		11.5		11.0
4-Ha	2.28	17.6	2.96	18.0	3.07	17.8	2.86	17.3	2.87	17.3
		2.6		3.0		3.3		2.9		3.6
3-H	3.14	11.7	2.93	11.0	3.33	11.3	3.07	11.5	3.15	11.0
		2.6		3.0		3.2		2.9		3.6
		2.6		3.0		3.2		2.9		3.6
11-H	4.01	2.6	4.31	3.0	4.37	3.2	4.13	2.9	3.92	(br s)
				0.5		1.5		1.6		
14-Ha	2.83	20.9	—		—		4.32	(s)	2.84	20.8
		8.2								8.3
14-Hb	2.24	20.9	—		—		—		2.24	20.8
		2.9								
13-H	3.43	8.2	3.28		3.47		3.46	(br s)	3.45	8.3
		2.9		2.0						
		1.9		3.0		2.9				
				0.5		1.5				
21-Ha	4.07	1.9	3.28	3.0	4.26	2.9	4.14	3.1	4.09	2.4
		—		10.5				—		—
21-Hb	—		2.93	2.0	—		—		—	
				10.5						
OCH_3	4.03	(s)	4.02	(s)	4.04	(s)	4.04	(s)	4.05	(s)
	4.02	(s)	3.93	(s)	3.94	(s)	4.03	(s)	4.00	(s)
NCH_3	2.32	(s)	2.43	(s)	2.47	(s)	2.48	(s)	2.33	(s)
CCH_3	1.99	(s)	2.15	(s)	2.17	(s)	2.00	(s)	1.95	(s)
	1.93	(s)	1.89	(s)	1.83	(s)	1.92	(s)	1.88	(s)
									0.91	(s)
$COCH_3$	2.25	(s)	2.26	(s)	2.25	(s)	2.26	(s)	2.12	(s)
Methylene group									2.93	17.8
									2.41	17.8

Chemical shift: In ppm downfield from internal TMS standatd, measured in $CDCl_3$.

J: Coupling constant in Hz.

Multiplicity under off-resonance condition; s=singlet, br s=broad signal.

observed fragment pattern. Therefore the structure of SG was considered to be 14-hydroxysaframycin A. The signal at 4.32 (14-Ha) of SG showed a coupling constant similar to the signal at 3.85 of SC, in which C-14 was substituted by methoxy group. Consequently, the 14-hydroxy group was con-

Table 2. ^{13}C NMR spectral data of newly prepared saframycins in comparison with those of saframycins A and D.

Carbon No.	Saframycins				
	A	D	F	G	H
$COCH_3$	196.7 (s)	195.8 (s)	195.8 (s)	196.8 (s)	211.6 (s)
C-15	186.5 (s)	153.3 (s)	153.4 (s)	188.3 (s)	186.5 (s)
C-5	185.2 (s)	186.1 (s)	185.7 (s)	185.2 (s)	185.5 (s)
C-18	183.4 (s)	139.4 (s)	141.1 (s)	182.7 (s)	183.7 (s)
C-8	180.8 (s)	181.2 (s)	180.6 (s)	180.7 (s)	180.7 (s)
NHCO	160.2 (s)	160.3 (s)	160.3 (s)	160.2 (s)	175.1 (s)
C-7	155.9 (s)	156.3 (s)	156.2 (s)	155.9 (s)	156.4 (s)
C-17	155.6 (s)	154.9 (s)	155.3 (s)		155.7 (s)
C-20	141.6 (s)	118.2 (s)		141.1 (s)	141.4 (s)
C-10	141.2 (s)	141.8 (s)	141.1 (s)	140.9 (s)	140.0 (s)
C-19	135.5 (s)	112.2 (s)	111.8 (s)	135.7 (s)	136.1 (s)
C-9	135.7 (s)	139.4 (s)	139.8 (s)		
C-16	129.2 (s)	118.2 (s)	119.3 (s)	129.1 (s)	129.0 (s)
C-6	128.3 (s)	127.5 (s)	127.9 (s)	128.3 (s)	127.2 (s)
CN	116.7 (s)	—	117.1 (s)	116.3 (s)	116.7 (s)
7,17-OCH_3	61.1 (q)	61.2 (q)	61.1 (q)	61.0 (q)	61.0 (q)
	61.0 (q)	61.0 (q)	60.8 (q)		
C-21	58.3 (d)	54.8 (t)	56.8 (d)	56.4 (d)	58.1 (d)
C-1	56.3 (d)	57.4 (d)	56.2 (d)	56.1 (d)	56.5 (d)
C-13	54.6 (d)	65.5 (d)	59.0 (d)	56.1 (d)	54.5 (d)
C-11	54.3 (d)	57.4 (d)	54.4 (d)	55.0 (d)	54.2 (d)
C-3	54.4 (d)	57.0 (d)	53.4 (d)	53.3 (d)	53.6 (d)
NCH_3	41.6 (q)	42.4 (q)	42.6 (q)	42.5 (q)	41.6 (q)
C-22	40.7 (t)	40.8 (t)	40.8 (t)	40.7 (t)	40.2 (t)
C-4	25.1 (t)	24.5 (t)	24.3 (t)	25.0 (t)	25.2 (t)
$COCH_3$	24.3 (q)	24.3 (q)	23.9 (q)		25.8 (q)
C-14	21.6 (t)	203.7 (s)	198.9 (s)	62.1 (d)	21.6 (t)
6,16-CH_3	8.7 (q)	8.9 (q)	9.3 (q)	8.7 (q)	8.5 (q)
		8.6 (q)	8.7 (q)		
$C(OH)CH_2$					74.4 (s)
CH_3					49.0 (t)
					31.3 (q)

Chemical shift: In ppm downfield from internal TMS standard, measured in $CDCl_3$.
Multiplicity under off-resonance condition; s=singlet, d=doublet, t=triplet, q=quartet.

sidered to be α-equatorial (Fig. 1).

Saframycin H (SH)

SH is a basic yellow compound giving needle which decomposes at 184~186°C. Results of elemental analysis and EI-MS data (*m/z* 620) suggest the molecular formula of SH to be $C_{32}H_{36}N_4O_9$. The formula indicates that SH has C_3H_6O atoms more than SA.

The UV and IR spectra of SH are similar to those of SA, indicating that SH has a basic structure similar to SA. However, when the ^{13}C NMR spectrum was compared with that of SA, new signals appeared at 74.4 (s), 49.0 (t) and 31.3 (q) which were not observed in SA (Table 2). Further, the signal at 196.7 (s) in SA was found to be shifted to a lower field of 211.6 (s).

In ^1H NMR spectrum of SH, new signals at 0.91 (3H, s), 2.93 (1H, d), 2.41 (1H, d) and 4.53 (1H, s) were observed. Those at 2.93 and 2.41 were noted to be as an AB doublet signal, having

a coupling constant of $J=17.8$ Hz. These data indicate the presence of an isolated methylene group with steric hindrance in the SH molecule. Since the signal at 4.53 was easily substituted by deuterium, the signal was ascribed to the hydroxyl proton. The above results indicate the presence of methyl, methylene and hydroxyl groups in the SH molecule. Actually the increased molecule of C_3H_6O was explained by the presence of the above three groups. Therefore, two possible partial structures, $CH_3C(OH)CH_2$ (a) and CH_3CCH_2OH (b) were considered. The spectral data of ^{13}C and ^1H NMR supported (b) as the most probable. The signal at 211.6 (s) is indicative of the presence of an isolated ketone structure. The fragmentation pattern of EI mass of the two antibiotics is similar and ^{13}C NMR spectral data indicate structural difference of the side chain between SA and SH. The presence of a fragmentation ion peak at m/z 562 in SH molecules suggests that loss of C_3H_6O from this molecule results in SA. The following two side chain structures were considered as candidates, $CH_2NHCOCH_2COHCOCH_3$ (c) and $CH_2NHCOC(OH)CH_2COCH_3$ (d). The presence of an m/z 562 fragment ion peak was explained by the retroaldol reaction, which led to the production of $CH_2NHCOCOCH_3$. Thus (d) appears the most reasonable structure and the structure of SH was finally determined as 25-dihydro-25-β-ketopropylsaframycin A (Fig. 1).

SF, SG and SH were shown to exhibit lower cytotoxic activities than SA against the cultured cell line of L1210 mouse leukemia[4] and ID_{50} (50% inhibition dose) values for SF, SG, SH and SA were 0.59, 0.03, 0.033 and 0.0056 μM, respectively.

Experimental

General

IR spectra were recorded in $CHCl_3$ solution using an IRA-2 spectrometer (Nihonbunko, Japan) and UV spectra were determined using a model 323 spectrophotometer (Hitachi, Japan). Field desorption (FD)-MS was determined using a JMS-O1SG-2 (Jeol, Japan), and EI-MS by an LKB 9000 (Shimadzu, Japan). ^1H NMR spectra (270 MHz) and ^{13}C NMR spectra were determined with a Jeol-GX270 (Jeol, Japan) and ^1H NMR spectrum (400 MHz) with JNM-GX400 (Jeol, Japan). Respective chemical shifts (in ppm) were recorded relative to internal TMS. Optical rotations were taken with a DIP-4 polarimeter (Nihonbunko, Japan). MP's were established using an MP-J2 (Yanagimoto, Japan) and are uncorrected. TLC by precoated silica gel of 0.5 mm thickness (Merck, Germany) was used. Silica gel of 70~230 mesh (Merck, Germany) was also implemented for column chromatography.

Antibiotic Production

Medium and other incubation conditions were the same as reported[2]. The only difference in this experiment was pH control of the culture at 6.0 to 6.5 after the midstationary phase by an automatic pH controller.

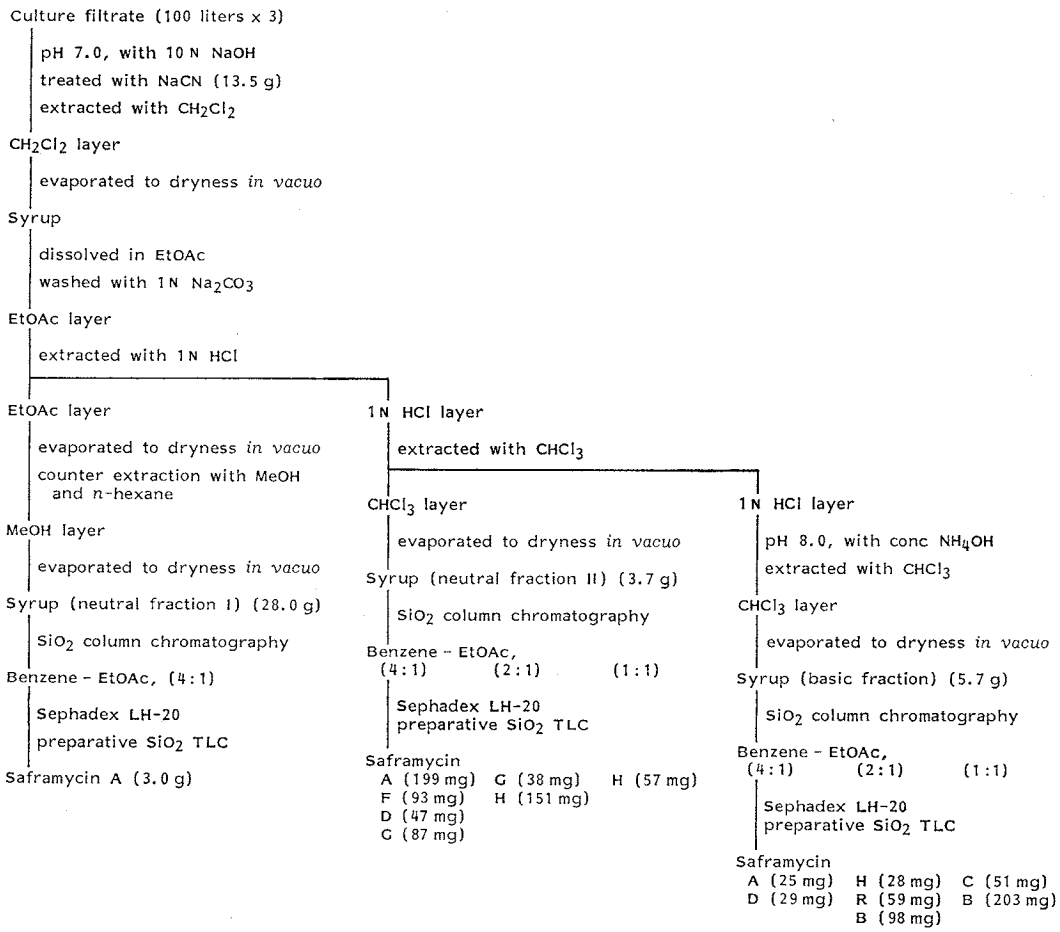
Isolation of SF, SG and SH

Culture filtrate (300 liters) of *Streptomyces lavendulae* No. 314 was adjusted to pH 7.0 and treated with 15 g of NaCN at 35°C. The detailed isolation procedures of saframycins are shown in Fig. 2.

Briefly, after incubation for 1 hour, the reaction mixture was extracted with 150 liters of dichloromethane. The solvent layer was evaporated *in vacuo* to dryness. The dried residue was dissolved in 1,000 ml of EtOAc. It was washed with 1 N Na_2CO_3 and extracted with 500 ml of 1 N HCl solution. The EtOAc layer was concd *in vacuo*. The dried residues were counter-extracted twice with 500 ml of 10% aq MeOH and *n*-hexane. The MeOH layer was collected and evaporated to dryness (neutral fraction I in Fig. 2). From this fraction saframycin A was the main substance obtained.

The 1 N HCl layer was extracted with $CHCl_3$ and the solvent layer was evaporated to syrup (neutral fraction II). After silica gel and Sephadex LH-20 column chromatography, the active fraction was

Fig. 2. Isolation procedures of saframycins.



further purified by preparative silica gel TLC. New saframycins were mainly obtained from this fraction.

The 1 N HCl layer referred to above was carefully adjusted to pH 8.0 with conc NH_4OH and extracted with $CHCl_3$ several times (basic fraction). The combined $CHCl_3$ layers were washed with 1 N Na_2CO_3 and then water. After dehydration, the solvent layer was evaporated. They were further purified by silica gel, Sephadex LH-20 and preparative TLC. SH was also obtained from this fraction.

The yields of saframycins from 300 liters culture broth were: 3,224 mg (SA), 301 mg (SB), 51 mg (SC), 76 mg (SD), 93 mg (SF), 125 mg (SG), 236 mg (SH) and 59 mg (SR).

Physico-chemical Properties of SF, SG and SH

SF: Slightly basic light yellow powder; mp 134~136°C (dec); $[\alpha]_D^{22}$ +28.4° (*c* 0.1, MeOH); elemental analysis, found: C 59.64, H 5.26, N 9.51, calcd for $C_{29}H_{30}N_4O_9$: C 60.20, H 5.23, N 9.86; UV λ_{max}^{MeOH} nm (log ε) 231 (3.98), 277 (4.11), 375 (3.65); IR ν^{CHCl_3} cm^{-1} 3550, 3400, 1720, 1690, 1660, 1620. ^1H NMR see Table 1. ^{13}C NMR see Table 2; EI mass *m/z* 578 (M^+), 478 (M^+-100), 236 (base).

SG: Slightly basic yellow powder; mp 134~136°C (dec); $[\alpha]_D^{22}$ −28.0° (*c* 1.0, MeOH); elemental analysis, found: C 60.68, H 5.55, N 9.45, calcd for $C_{29}H_{30}N_4O_9$: C 60.20, H 5.23, N 9.68; UV λ_{max}^{MeOH} nm (log ε) 266 (4.19); IR ν^{CHCl_3} cm^{-1} 3560, 3400, 1685, 1655, 1610. ^1H NMR see Table 1. ^{13}C NMR see Table 2; EI mass *m/z* 578 (M^+), 478 (M^+-100), 234 (base).

SH: Slightly basic light yellow needles; mp 184~186°C (dec); $[\alpha]_D^{22}$ −7° (*c* 0.1, MeOH); elemental

analysis, found: C 61.68, H 5.84, N 8.71, calcd for $C_{32}H_{36}N_4O_9$: C 61.92, H 5.85, N 9.03; UV λ_{max}^{MeOH} nm (log ε) 268 (4.19); IR ν^{CHCl_3} cm^{-1} 3420, 1675, 1655, 1610. ^1H NMR see Table 1. ^{13}C NMR see Table 2; EI mass *m/z* 620 (M^+), 562 (M^+-58), 462 (M^+-158), 243, 220 (base).

Acetylation of SG

SG (120 mg, 0.5 mM) was dissolved in 5 ml of pyridine and 0.5 ml of acetic anhydride was added and mixed. The solution was allowed to stand at room temp overnight. The residue obtained by evaporation was dissolved in 100 ml of EtOAc and washed with 0.05 N $NaCO_3$. After washing with water, the EtOAc layer was evaporated to dryness. The residue was subjected to preparative TLC using EtOAc - benzene (2:1) as a solvent. The yield of purified SG-monoacetate was 89 mg. SG monoacetate was crystallized from ether.

Physico-chemical Properties of SG Acetate

SG-Monoacetate: MP 160°C (dec); elemental analysis, found: C 59.47, H 5.32, N 8.79, calcd for $C_{31}H_{32}N_4O_{10}$: C 59.99, H 5.20, N 9.03; UV λ_{max}^{MeOH} nm (log ε) 266 (41.6); IR λ^{CHCl_3} cm^{-1} 3400, 1730, 1680, 1655, 1615. ^1H NMR (in $CDCl_3$) δ 2.08 (3H, s), 5.40 (1H, s); mass *m/z* 620 (M^+, 1.0), 562 ($M^+-CH_2CO_2$, base), 462 ($M^+-CH_2CO_2-100$, 60).

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