BACTERIAL PRODUCTION OF 7-FORMAMIDOCEPHALOSPORINS

ISOLATION AND STRUCTURE DETERMINATION

PUSHPA D. SINGH, MARIAN G. YOUNG, JANICE H. JOHNSON,
CHRISTOPHER M. CIMARUSTI and RICHARD B. SYKES

The Squibb Institute for Medical Research
P. O. Box 4000
Princeton, New Jersey 08540, U.S.A.

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Two new 7-formamidocephalosporins have been isolated as their acetyl derivatives (SQ 28,516 and SQ 28,517) from fermentations of a *Flavobacterium* sp. SC 12,154. Structure **1** was deduced for SQ 28,516 from its spectroscopic properties while structure **2** was proposed for SQ 28,517. SQ 28,516 exhibits weak antibacterial activity.

The production of β-lactam antibiotics by bacteria has been recently reported; these include monobactams[1~9], a carbapenem[10] and a cephalosporin[11]. During a screening program developed to detect β-lactam antibiotics produced by bacteria[8], we isolated a strain of *Flavobacterium* sp. that produces deacetoxycephalosporin C[11] and a mixture of novel 7-substituted cephalosporins. A description of the producing strain and its fermentation conditions has been reported previously[11]. This paper describes the isolation and structure determination of two cephalosporins, SQ 28,516 (**1**) and SQ 28,517 (**2**) (Fig. 1). The biological properties of SQ 28,516 are also presented.

Isolation Procedure

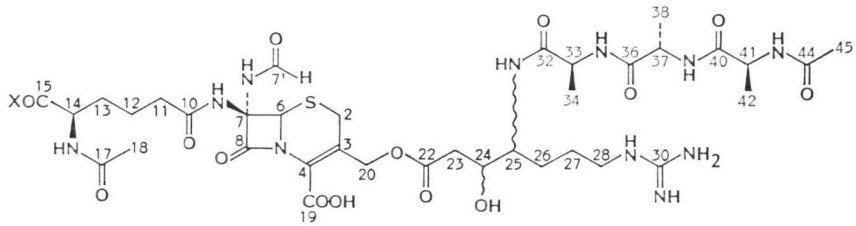
The fermentation of *Flavobacterium* sp. SC 12,154 produced a mixture of cephalosporins; deacetoxycephalosporin C was found in the cells[11] whereas a mixture of 7-substituted cephalosporins was present in the broth supernate. Antibiotic production in the fermentation broth and the isolation of the 7-substituted cephalosporins, as their acetyl derivatives, were monitored using *Bacillus licheniformis* (SC 9262) as the test organism. The isolation and purification procedure is outlined in Fig. 2.

The fermentation broth contained a mixture of antibiotics which were cationic at pH 2.3~7.0 and anionic at pH 9.2. The antibiotics themselves were heat and pH labile, and all attempts to isolate them were unsuccessful. However, their acetyl derivatives were somewhat more stable and the antibiotics were therefore isolated as their acetyl derivatives. The fermentation broth was centrifuged, the supernate adjusted to pH 4.0 and the bioactive components were absorbed on a strongly acidic ion-exchange resin and acylated with acetic anhydride. The acetyl derivatives, SQ 28,516 and SQ 28,517, were further purified by a combination of adsorption, ion-exchange and reverse-phase chromatography.

SQ 28,516

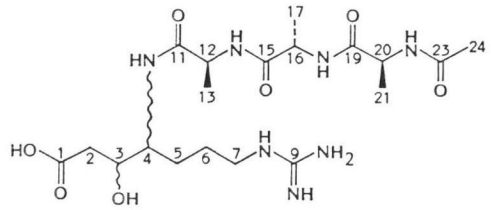
The major antibiotic of the fermentation, isolated as the *N,N'*-diacetyl derivative, SQ 28,516 (**1**), is a water soluble, amphoteric cephalosporin (electrophoresis data in Table 1), $[\alpha]_D^{25}$ $-2.6°$ (*c* 1.05, H_2O). The UV spectrum showed a λ_{max} at 263 nm ($E_{1cm}^{1\%}$ 69) and the IR spectrum in KBr exhibited bands at 1770 (β-lactam carbonyl), 1720 (ester) and 1650 (secondary amide) cm^{-1}. The spectral data, taken together with the origin of SQ 28,516 in a screen specific for β-lactam antibiotics, strongly suggested a

Fig. 1. Structures of SQ 28,516, SQ 28,517 and the hydrolysis product (**3**).



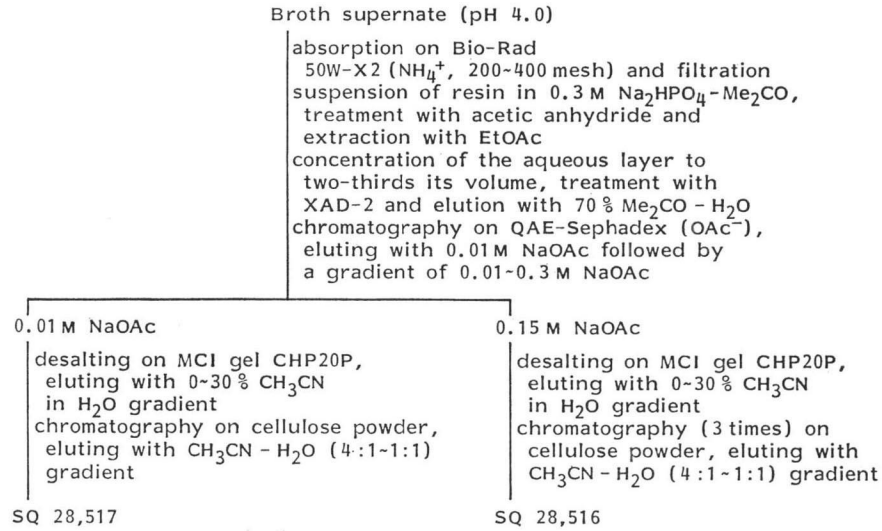
1 SQ 28,516 X = OH

2 SQ 28,517 X = NH_2



3
(Hydrolysis product)

Fig. 2. Isolation of SQ 28,516 and SQ 28,517.



cephalosporin derivative.

Analysis of the acid hydrolysate (6 N HCl, 110°C, 15 hours) by dansylation[12] followed by TLC analysis on polyamide plates, indicated the presence of alanine, glycine, α-aminoadipic acid and several unidentified components. The configurations of alanine and α-aminoadipic acid, as the 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate[13] (GITC) derivatives, were determined by HPLC using peak enhancement with authentic samples, and were shown to be L and D, respectively. The presence of glycine[14] and D-α-aminoadipic acid in this hydrolysate was consistent with the formulation of SQ 28,516 as a cephalosporin derivative.

Table 1. Electrophoresis of SQ 28,516, SQ 28,517 and the hydrolysis product (**3**).

Buffer (pH)	Mobility[a]		
	SQ 28,516	SQ 28,517	**3**
Sodium 0.05 M phosphate (2.3)	−0.17	−0.43	−0.48
Sodium 0.05 M phosphate (4.5)	+0.24	−0.23	−0.49
Sodium 0.05 M phosphate (7.0)	+0.25	0.00	0.00
Sodium 0.05 M carbonate (9.2)	+0.24	0.00	0.00

[a] On Whatman No. 1 paper, 12 V/cm, 1 hour; mobilities relative to vitamin B_{12} (0.00) and *p*-nitrobenzenesulfonate anion (1.00).

Table 2. ^{1}H NMR data for SQ 28,516, SQ 28,517 and the hydrolysis product (**3**).

Position No.	SQ 28,516 δ(J Hz)[a]	SQ 28,517 δ(J Hz)[a]	Position No.	**3** δ(J Hz)[a]
34, 38, 42	1.30 (m)	1.30 (m)	13, 17, 21	1.25 (m)
26, 27	1.40~1.80 (m)	1.40~1.85 (m)	5, 6	1.40~1.52 (m)
12, 13	1.60 (m)	1.60 (m)	24	1.70 (s)
18, 45	1.95 (s)	1.95 (s)	2	2.15 (8.0, 16.0); 2.31 (4.0, 16.0)
11	2.30 (m)	2.30 (m)	7	3.08 (m)
23	2.40 (8.0, 16.0); 2.60 (4.0, 16.0)	2.40 (8.0, 16.0); 2.60 (4.0, 16.0)	4	3.70 (m)
28	3.10 (m)	3.10 (m)	3	3.85 (m)
2	3.21 (17.7); 3.55 (17.7)	3.21 (17.7); 3.55 (17.7)	12, 16, 20	4.15 (m)
25	3.70 (m)	3.70 (m)		
24	3.90 (m)	3.90 (m)		
14	4.08 (m)	4.10 (m)		
33, 37, 41	4.15 (m)	4.15 (m)		
20	4.62 (12.8); 4.78 (12.8)	4.70 (12.8); 4.74 (12.8)		
6	5.25 (s)	5.25 (s)		
7′	8.05 (s)	8.00 (s)		

[a] ppm downfield from TMS using HDO (4.73 ppm) as an internal standard.

The molecular weight and empirical formula of SQ 28,516 were determined by fast atom bombardment (FAB)[15] mass spectrometry. Both positive and negative ion spectra implied molecular weights of 913 and 935 for the free acid and the monosodium salt, respectively. The exact mass observed for $C_{36}H_{56}N_{11}O_{15}S$ (M+H) was 914.362 (theory 914.367). Since SQ 28,516 was subsequently shown to be an *N*,*N*′-diacetyl derivative, its parent antibiotic, which must have a molecular weight of 829, would be the most complex cephalosporin isolated to date.

The ^{1}H NMR data for SQ 28,516 and assignments made with the aid of decoupling experiments are given in Table 2. The appearance of the peak assigned to the C-6 proton (singlet at δ 5.25) implied a 7,7-disubstituted moiety; however no methoxyl singlet was evident, eliminating cephamycins from consideration. The lack of a signal in the ^{13}C NMR spectrum (*ca.* 96 ppm)[16] attributable to a 7-acylamino-7-oxygenated carbon, further indicated that SQ 28,516 was not a 7-alkoxy or acyloxy cephalosporin. The conjunction of peaks at 8.05 ppm in the ^{1}H NMR spectrum and 77.8 ppm in the ^{13}C NMR spectrum strongly suggested a 7-acylamino-7-formamidocephalosporin.

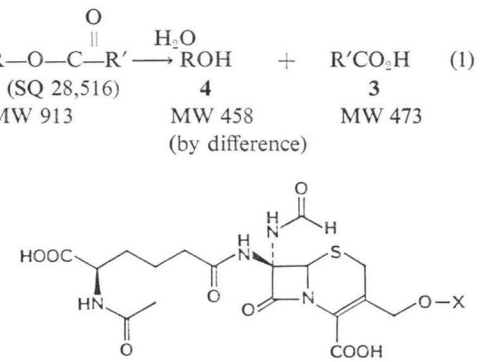
Table 3. ^{13}C NMR data for SQ 28,516.

Chemical shifts (δ)[a]	Carbon type[b]	Assignment
17.5 17.7 (2C)	CH_3	C-34, C-38, C-42
22.8 23.2	CH_3	C-18, C-45
22.7 25.6 26.7 27.6	CH_2	C-12, C-13, C-26, C-27
32.3 35.8 39.8 41.8	CH_2	C-2, C-11, C-23, C-28
50.8 50.9 51.3 54.2 55.9	CH	C-14, C-25, C-33, C-37, C-41
64.1 65.1	Not clear	C-6, C-20
70.9	〃	C-24
77.8	〃	C-7
115.3	C	C-3
132.9	C	C-4
157.3 159.7 163.9 168.5 173.7 173.9 174.7 175.1 175.2 175.4 177.9 179.2	C=O	C-7′, C-8, C-10, C-15, C-17, C-19, C-22, C-30, C-32, C-36, C-40, C-44

[a] ppm downfield from TMS, using dioxane (67.6 ppm) as an internal standard.

[b] Assignments are made by the INEPT technique.

Fig. 3. Hydrolysis of SQ 28,516.



Though unreported as natural products, 7α-formamidocephalosporins have recently been prepared synthetically[17]. The chemical shifts reported for the C-6 and C-7′ protons were a striking match for those of SQ 28,516.

The ^{13}C NMR spectrum of 7α-formamido-7β-phenylacylaminocephalosporanic acid (prepared using the procedure of MILNER[17]) exhibited a peak at δ 79.1 assigned to C-7. The correspondence in chemical shifts of the C-7 carbon of this material and SQ 28,516, together with the data discussed above, led us to assign a 7α-formamido structure to SQ 28,516 and its parent antibiotic. The complete structural assignment of SQ 28,516 was based on the following consideration.

^1H NMR decoupling experiments indicated that the alanyl methyl protons (δ 1.30) were coupled to the alanyl methine protons (δ 4.15); the C-14 proton (δ 4.08) was coupled to the C-13 protons (δ 1.60); the C-11 protons (δ 2.30) were coupled to the C-12 protons (δ 1.60); the C-25 proton (δ 3.70) was coupled to the C-24 proton (δ 3.90) and the protons at C-26 (δ 1.40 ~ 1.80); and the C-28 protons (δ 3.10) were coupled to the C-27 protons (δ 1.40 ~ 1.80).

The ^{13}C NMR data and assignments are presented in Table 3. The multiplicities of the carbons were determined using the INEPT[18] technique.

Decomposition (*via* hydrolysis) of SQ 28,516 was facile and varying amounts of a hydrolysis product (**3**) were obtained during isolation and upon storage, even at $-20°C$. The hydrolysis product (**3**) is cationic at pH 2.3 and 4.5 and uncharged at pH 7.0 and 9.2 (Table 1), has an Rf of 0.15 on silica gel (Analtech GH 250, CH_3CN - H_2O, 7: 3) and is positive in both the RYDON-SMITH and SAKAGUCHI tests but negative in the ninhydrin test. SQ 28,516, itself, has an Rf of 0.49 on silica gel (Analtech GH 250, CH_3CN - H_2O, 7: 3) and is positive in both the RYDON-SMITH and SAKAGUCHI tests but negative in the

ninhydrin test.

The molecular weight and empirical formula of the hydrolysis product (**3**) were determined by FAB-MS[15]. Both positive and negative ion spectra indicated molecular weights of 473 and 495 for the free acid and the sodium salt, respectively. The exact mass observed for $C_{19}H_{36}N_7O_7$ (M+H) was 474.268 (theory 474.268). If our formulation of the origin of this hydrolysis product is correct, it should represent R′CO_2H of equation 1 (Fig. 3). Correspondingly, the ROH portion should then be *N*-acetyl-7α-formamidodeacetylcephalosporanic acid (**4**). The correspondence of the molecular weight of **4** with that calculated strongly supports the formulation of SQ 28,516 as discussed herein.

The ^{1}H NMR data for the hydrolysis product (**3**) is presented in Table 2. Decoupling experiments indicated that the alanyl methyl protons (δ 1.25) were coupled to the alanyl methine protons (δ 4.15); the C-2 protons (δ 2.15, 2.31) were coupled to each other and to the proton at C-3 (δ 3.85); the C-3 proton (δ 3.85) was coupled to the C-2 protons (δ 2.15, 2.31) and to the C-4 proton (δ 3.70); the C-4 proton (δ 3.70) was coupled to the C-5 protons (δ 1.40~1.52); and the protons at C-7 (δ 3.08) were coupled to the protons at C-6 (δ 1.40~1.52).

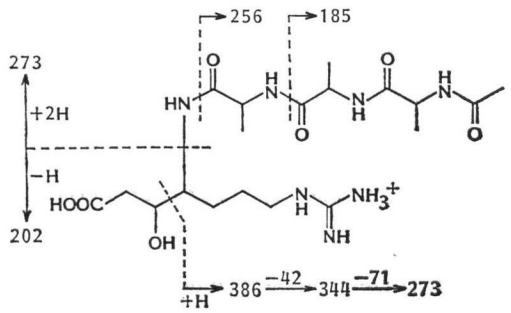
The sequence of amino acids in the hydrolysis product (**3**) was determined by FAB-MS. A mass analyzed ion kinetic energy (MIKE) spectrum[19] of the peak at *m/z* 474 $(M+H)^+$ resulted in fragment ions at *m/z* 386, 344, 273, 256, 202 and 185 establishing the sequence shown in Fig. 4.

Acid hydrolysis of compound **3** in 6 N HCl at 110°C for 15 hours gave a mixture that was separated by ion-exchange chromatography into alanine and 3-hydroxy-4-amino-7-guanidoheptanoic acid. Alanine was assigned the L-configuration by peak enhancement on HPLC with authentic alanine-GITC[13] derivative. The identity of 3-hydroxy-4-amino-7-guanidoheptanoic acid was established by ^{1}H NMR and FAB-MS. A multiplet at δ 4.20 (pH 1.0) was attributed to the methine proton at C-3 (unaffected by a change in pH), a multiplet at δ 3.30 (pH 1.0) was attributed to the methine proton at C-4 (shifted to δ 2.65 at pH 11.0), and a multiplet at δ 3.10 (pH 1.0~11.0) was attributed to the C-7 protons. Both positive and negative ion FAB-MS led to the conclusion that the molecular weight was 218 for the free acid.

SQ 28,517

A minor component of the fermentation, SQ 28,517 (**2**), a water soluble 7-substituted cephalosporin, was also isolated. In contrast to SQ 28,516, SQ 28,517 is cationic at pH 2.3 and 4.5 and is uncharged

Fig. 4. Mass spectrometric fragmentation of the hydrolysis product (**3**).



3

Table 4. Antibacterial activity of SQ 28,516.

Organism	SC No.	MIC (μg/ml)[a]
Escherichia coli	12155	100
E. coli	8294	>800
E. coli	10857	>800
E. coli	10896	>800
E. coli	10909	>800
Proteus mirabilis	3855	>800
P. rettgeri	8479	>800
Staphylococcus aureus	1276	>800
Streptococcus agalactiae	9287	400
Micrococcus luteus	2495	25

[a] Susceptibility determined by microtiter assay at 10^3 cfu/ml in AA broth (BBL).

at pH 7.0 and 9.2 (Table 1). SQ 28,517 has an Rf of 0.3 on silica gel (Analtech GH 250, CH_3CN - H_2O, 7: 3) and is positive in the RYDON-SMITH and SAKAGUCHI tests and negative in the ninhydrin test. Analysis of the acid hydrolysate (6 N HCl, 110°C, 15 hours) by dansylation[13] followed by TLC analysis on polyamide plates indicated the presence of alanine, glycine, α-aminoadipic acid and several unidentified products.

The positive-ion FAB-MS of SQ 28,517 did not give a parent ion. The UV spectrum showed a λ_{max} at 265 nm ($E^{1\%}_{1cm}$ 33) and the IR spectrum in KBr exhibited bands at 1760 (β-lactam carbonyl), 1720 (ester) and 1650 (secondary amide) cm^{-1}.

The ^1H NMR data for SQ 28,517 is given in Table 2, and is similar to the data for SQ 28,516. The chemical shift of the β-lactam proton at C-6 (δ 5.25) and the C-7′ proton (δ 8.00) are analogous to those observed for SQ 28,516.

The C-20 side chain of SQ 28,517 was shown to be the same as that in SQ 28,516, by the analysis described above. Structure **2** is consistent with the spectroscopic and electrophoretic data presented in this paper, and is thus a reasonable candidate for SQ 28,517.

Conclusion

In conclusion, two novel 7-formamidocephalosporins have been isolated as their *N,N′*-diacetyl derivatives (SQ 28,516 and SQ 28,517) from a bacterial culture. SQ 28,516 exhibits weak antibacterial activity (Table 4). To the best of our knowledge, these constitute the most complex naturally-produced cephalosporins isolated to date. The same bacterial culture produces deacetoxycephalosporin C[11]. The origin of the nitrogen at C-7 in SQ 28,516 and SQ 28,517 poses an interesting biosynthetic problem. Also, whether deacetoxycephalosporin C and the 7-formamidocephalosporins are biosynthetically related is unknown. With the discovery of monobactams[1~9], a carbapenem[10], and cephalosporins[11] of bacterial origin, it is obvious that bacteria have the biosynthetic capability to produce a range of β-lactam-containing structures at least comparable to those produced by streptomycetes and fungi.

Experimental

NMR spectra were determined on a Joel Ltd. GX 400 spectrometer. IR spectra were recorded on a Perkin-Elmer model 621 spectrometer. Rotations were measured on a Perkin-Elmer model 141 polarimeter. Mass spectra were determined on a VG Analytical Ltd. model ZAB 1F spectrometer.

Isolation of SQ 28,516 and SQ 28,517

At harvest, the cells from a 20-liter fermentation of *Flavobacterium* sp. SC 12,154 were separated by centrifugation. The pH of the broth supernate (20 liters) was adjusted to 4.0 with HCl and the bioactive components were absorbed onto ion-exchange resin Bio-Rad 50W-X2 (NH_4^+, 200~400 mesh, 350 g, batch process, 2 hours). The resin was filtered, washed with H_2O (1 liter) and suspended in a mixture of 0.3 M Na_2HPO_4 (pH 8.2, 2.5 liters) and acetone (250 ml). Aliquots of acetic anhydride (7.5 ml) were added to the suspension with stirring (four times at 40-minute intervals) and the pH of the reaction mixture was maintained at about 8 with 3 N NaOH. After 2 hours of stirring, the resin was removed by filtration and washed with H_2O (500 ml). The aqueous solution was extracted with EtOAc (2×2 liters). The EtOAc layer was discarded and the aqueous layer was concentrated to two-thirds its volume and stirred with Amberlite XAD-2 resin (500 ml, 2 hours). The resin was filtered, washed with H_2O (1 liter) and packed in a column. The bioactive components were eluted with 70% $(CH_3)_2CO$ - H_2O (3 liters). The bioactive fractions were combined and the solvents were removed under reduced pressure (temperature <35°C). The residue (1.28 g) was dissolved in H_2O (10 ml) and purified on an ion-exchange column of Sephadex QAE (OAc^-, 200~400 mesh, 2.5×17 cm) that had been equilibrated with 0.01 M NaOAc. The column was eluted with 0.01 M NaOAc (500 ml) followed by a gradient of 0.01~0.3 M NaOAc (1.5 liters). SQ 28,517 was not retained on the resin and eluted with 0.01 M NaOAc whereas

SQ 28,516 was retained on the resin and eluted at 0.15 M NaOAc.

The bioactive fractions containing SQ 28,517 (200 ml) were desalted on a column of MCI gel CHP-20P (2.5×20 cm). The column was washed with H_2O (400 ml) and the activity was eluted with a gradient of 0~30% CH_3CN - H_2O (800 ml). The bioactive fractions were combined and concentrated. The residue (108 mg) was further purified on a column of cellulose (2.5×20 cm) eluting with a gradient of 4: 1~1: 1, CH_3CN - H_2O (800 ml). The bioactive fractions were combined and concentrated to give chromatographically homogeneous SQ 28,517 (6.3 mg) as an off-white foam.

The bioactive fractions containing SQ 28,516 (250 ml), from the Sephadex QAE column, were desalted on a column of MCI gel CHP20P (2.5×25 cm). The column was washed with H_2O (450 ml) and then eluted with a gradient of 0~30% CH_3CN - H_2O (800 ml). The bioactive fractions were combined and concentrated to dryness. The residue (116 mg) was further purified on a cellulose column (2.5×24.5 cm) eluting with a gradient of 4: 1~1: 1, CH_3CN - H_2O (1.2 liters). The bioactive fractions were combined and concentrated to dryness (20.2 mg). The cellulose chromatography was repeated twice on the 20.2 mg sample using the same gradient, to give chromatographically homogeneous SQ 28,516 (16.2 mg) as an off-white foam.

Isolation of Hydrolysis Product (**3**)

After storage for four months at −20°C, TLC of a sample of SQ 28,516 showed the presence of hydrolysis product (**3**). This sample (39.6 mg) was chromatographed on a cellulose column (2.5×25 cm), eluting with a gradient of 4: 1~1: 1, CH_3CN - H_2O (1.2 liters), to yield 24.2 mg of SQ 28,516 and 11.5 mg of hydrolysis product (**3**).

Hydrolysis of **3**

A solution of 7.75 mg of the hydrolysis product (**3**) in 1.5 ml of 6 N HCl was heated at 110°C for 15 hours and then concentrated *in vacuo*. The residue was chromatographed on a 1.1×16 cm column of Dowex 50W-X2 resin (200~400 mesh, pyridinium form) eluting with a linear gradient of 0.2~2.0 M pyridinium acetate (pH 5.1), to give alanine (4.7 mg) and 3-hydroxy-4-amino-7-guanidoheptanoic acid (2.8 mg). ^1H NMR of 3-hydroxy-4-amino-7-guanidoheptanoic acid (D_2O, pH 7): δ 1.50~1.70 (m), 2.35 (t), 3.15 (m) and 4.15 (m).

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