STRUCTURAL INVESTIGATION OF THE ANTIBIOTIC SPORAVIRIDIN

XIV.† ISOLATION OF COMPONENTS OF INTACT SPORAVIRIDIN††

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Six components of sporaviridin (SVD) were successfully isolated by HPLC using methanol - 1 M ammonium chloride as the mobile phase. Each component possesses expectedly antimicrobial activity. To ensure the structural relationship between the intact SVD and *N*-acetylated sporaviridins (*N*-Ac-SVD), they were converted to the corresponding of *N*-acetates by acetylation in methanol, whose structures had already been determined as glycosides consisting of a 34-membered macrocyclic lactone, viridopentaoses, D-glucose and *N*-acetylvancosamine.

Sporaviridins (abbreviated as SVD) are basic glycoside antibiotics produced by *Streptosporangium viridogriseum*[2]. They exhibit strong inhibitory activity against Gram-positive bacteria, acid fast bacteria and trichophyton, but also show considerable toxicity such as hemolysis and fish poisoning. Although various structural studies have been attempted since they were isolated in 1963, their structures have remained unsolved. Because the intact antibiotics are basic and water-soluble, but very labile under basic conditions, we carried out the isolation[3] and the structural determination using their *N*-acetylated sporaviridins (*N*-Ac-SVD), of which the structures have recently been proposed as shown in Fig. 1[1]. They are composed of six components and each has a 34-membered lactone and seven monosaccharide units in which a pentasaccharide, viridopentaose[4], and two monosaccharides are included. The structural differences of the six components of *N*-Ac-SVD are due to methyl or ethyl group at C-2 and three viridopentaose A, B or C at C-13. However, these *N*-acetylated derivatives do not show antimicrobial activity.

Therefore, free bases of the intact antibiotics were required and had to be separated to the individual components which are closely related to one another in both of chemical and physical properties, and molecular weights. In this paper we describe the isolation and physico-chemical properties of the intact SVD obtained.

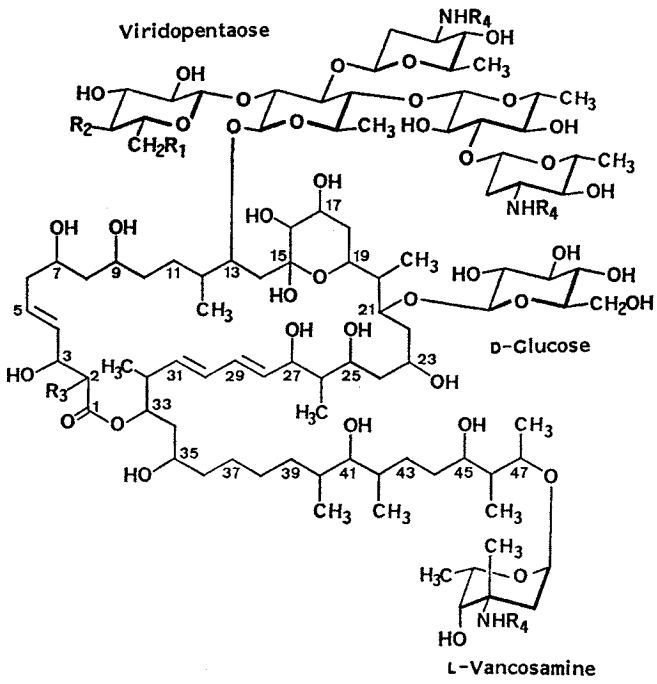
Isolation of SVD Components

During isolation and structural determination of *N*-Ac-SVD, we found that they are unstable under basic conditions and the characteristic behavior was used for the degradative studies. Treatment of *N*-Ac-SVD with a base such as aqueous ammonia and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) resulted in the cleavage of a glycosidic bond to liberate the pseudoaglycones (*N*-Ac-pAG) and the constituent pentasaccharides, viridopentaoses[4,5]. These degradation products were key com-

† See ref 1.

†† This paper is dedicated to Professor KENNETH L. RINEHART in honor of his 60th birthday.

Fig. 1. Structures of SVD and *N*-Ac-SVD.



SVD-A_1	R_1=H	R_2=OH	R_3=C_2H_5	R_4=H
SVD-A_2	R_1=H	R_2=OH	R_3=CH_3	R_4=H
SVD-B_1	R_1=H	R_2=NH_2	R_3=C_2H_5	R_4=H
SVD-B_2	R_1=H	R_2=NH_2	R_3=CH_3	R_4=H
SVD-C_1	R_1=OH	R_2=OH	R_3=C_2H_5	R_4=H
SVD-C_2	R_1=OH	R_2=OH	R_3=CH_3	R_4=H
N-Ac-SVD-A_1	R_1=H	R_2=OH	R_3=C_2H_5	R_4=Ac
N-Ac-SVD A_2	R_1=H	R_2=OH	R_3=CH_3	R_4=Ac
N-Ac-SVD-B_1	R_1=H	R_2=NHAc	R_3=C_2H_5	R_4=Ac
N-Ac-SVD-B_2	R_1=H	R_2=NHAc	R_3=CH_3	R_4=Ac
N-Ac-SVD-C_1	R_1=OH	R_2=OH	R_3=C_2H_5	R_4=Ac
N-Ac-SVD-C_2	R_1=OH	R_2=OH	R_3=CH_3	R_4=Ac

pounds in determining the total structures of *N*-Ac-SVD. This chemical transformation is more facile in the case of the intact SVD. For instance, an aqueous solution of SVD has pH 9 and half life times in aqueous and methanol solutions were about 2 days and 1 week, respectively. So they decompose quickly to their degradation products due to their basicity in aqueous and methanol solution.

For these reasons, a mobile phase of the HPLC for separation of SVD components had to be carefully chosen. We have already shown that the reversed phase HPLC using an ammonium chloride-containing mobile phase is suitable for analysis of aculeximycin which is also a basic glycoside antibiotic and is similar to SVD[6]. The stability of SVD was examined in the mobile phase, methanol - 1 M ammonium chloride. SVD in this mobile phase has proven to be considerably more stable than in aqueous and methanol, indicating that this solvent system depresses the chemical decomposition of SVD and is suitable for our objective. After the optimization of the operating conditions for the preparative HPLC, they were fixed as follows: Column; ODS-silica gel, mobile phase; methanol - 1 M ammonium chloride (70 : 30), flow rate; 2 ml/minute, detection; UV 232 nm.

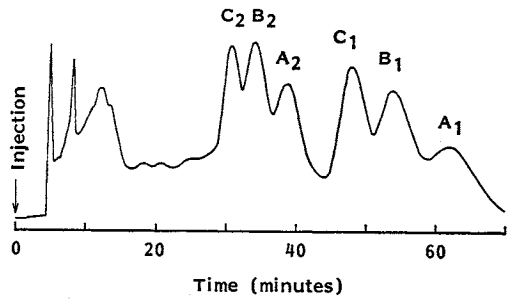
Fig. 2 shows the HPLC of SVD components under the preparative HPLC conditions. Although this is not a base-line separation among the components, the six components can be resolved to considerable extent. Several peaks around the retention time of 10 minutes were found later to be the pseudoaglycones which were produced by the cleavage of the glycosidic bond at C-13 of the aglycones. The preparative HPLC of the mixture was repeatedly carried out under the chromatographic conditions. Since the resulting fractions contained the nonvolatile inorganic salt together with the desired antibiotics, a desalting operation was required. For this purpose, commercially available C18 cartridge such as BOND ELUT C18 and SEP-PAK C18 (two cartridges connected in series in this case) were very effective. Finally we were able to obtain the intact SVD-A_1 (7.8 mg), A_2 (13.1 mg), B_1 (12.4 mg), B_2 (10.0 mg), C_1 (21.8 mg) and C_2 (10.2 mg) from 370 mg of the mixture.

Physico-chemical Properties of SVD Components

The physico-chemical properties of each component thus obtained are summarized in Table 1. They were obtained as amorphous white powders and are closely similar to one another. The fast atom bombardment mass spectrometry (FAB-MS) gave firm molecular weight information. Fig. 3 demonstrates the FAB-MS of SVD-C_1 and C_2 as the representative samples. The protonated molecule, $(M+H)^+$, is observed at *m/z* 2,095 as the most abundant peak in this region of the spectrum of SVD-C_1 using glycerol as the matrix, indicating that it has the molecular weight of 2,093. Other prominent ions at *m/z* 1,364 and 729 are considered to correspond to the pseudoaglycone and the constituent pentasaccharide (deacetylated viridopentaose A, DA-VP-A) ions. Analogously, the $(M+H)^+$ appears most abundantly at *m/z* 2,081 in the molecular ion region together with the pseudoaglycone at *m/z* 1,350 and DA-VP-A at *m/z* 729 in the spectrum of SVD-C_2.

The UV spectra of the six components show an absorption maximum at 232 nm, which support the presence of a conjugated diene system in their molecules. The two characteristic bands, hydroxy and amino groups at 3700~3000 cm^{-1} and ester carbonyl at 1700 cm^{-1} are in common observed in the IR spectra. The ^1H NMR spectra of SVD-A_1 and A_2 are shown

Fig. 2. HPLC of SVD components under the preparative conditions.

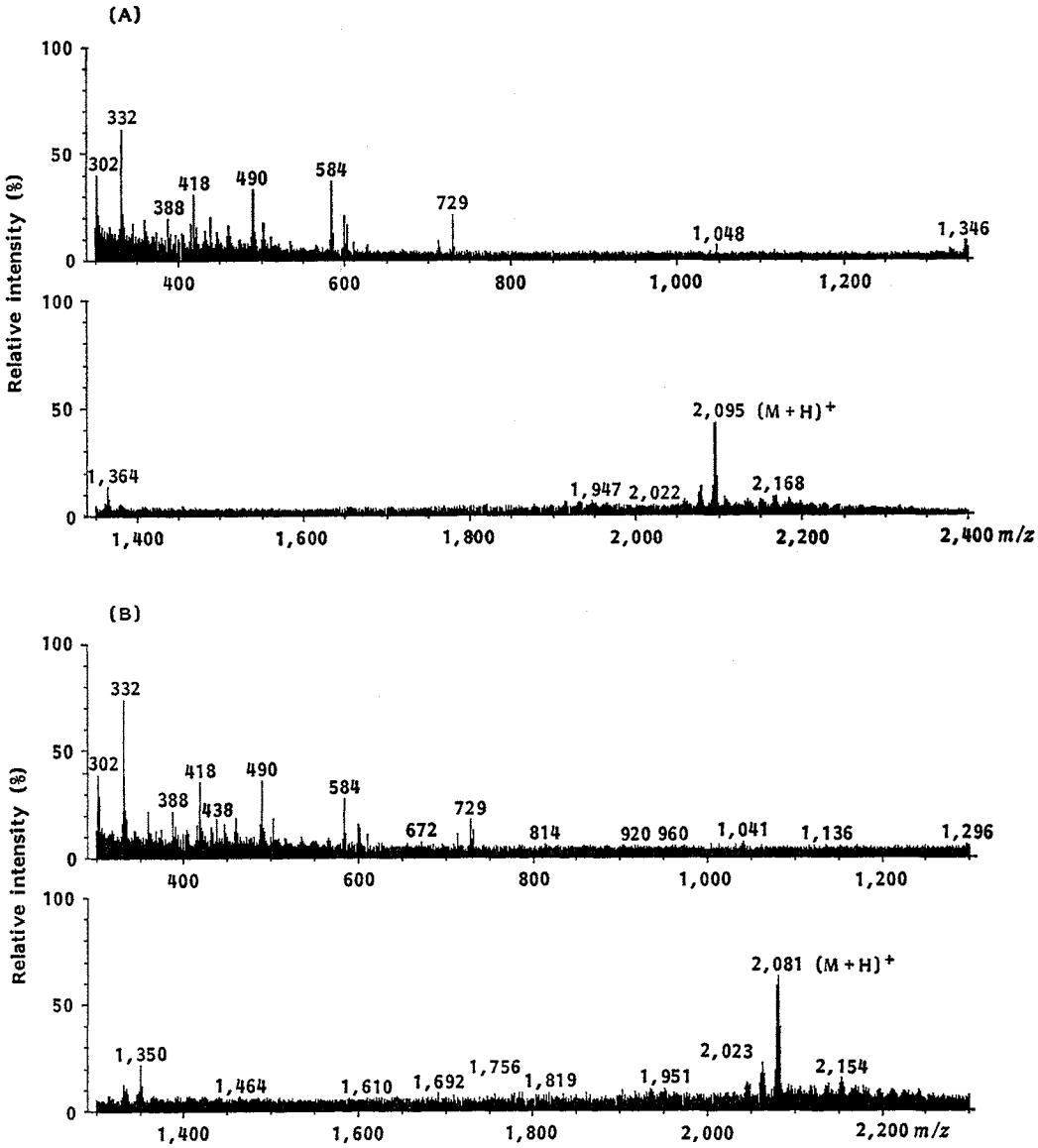


Column: Cosmosil 5C18-P (10×250 mm), mobile phase: MeOH - 1 M NH_4Cl (73:27), flow rate: 2 ml/minute, detection: UV 232 nm.

Table 1. Physico-chemical properties of SVD.

	A_1	A_2	B_1	B_2	C_1	C_2
Appearance	White powder	White powder	White powder	White powder	White powder	White powder
MP (°C)	190~192	190~193	196~199	191~193	194~196	196~198
FAB-MS (*m/z*)	2,079 $(M+H)^+$	2,065 $(M+H)^+$	2,078 $(M+H)^+$	2,064 $(M+H)^+$	2,095 $(M+H)^+$	2,081 $(M+H)^+$
Molecular formula	$C_{100}H_{179}N_3O_{41}$	$C_{99}H_{177}N_3O_{41}$	$C_{100}H_{180}N_4O_{40}$	$C_{99}H_{178}N_4O_{40}$	$C_{100}H_{179}N_3O_{42}$	$C_{99}H_{177}N_3O_{42}$
$[\alpha]_D^{25}$ (MeOH)	−8.8° (*c* 0.41)	−9.6° (*c* 0.57)	−13.6° (*c* 0.56)	−10.0° (*c* 0.66)	−13.5° (*c* 0.97)	−8.0° (*c* 0.48)
UV λ_{max}^{EtOH} (log ε)	232(3.94)	232 (4.66)	232 (4.49)	232 (4.40)	232 (4.20)	232 (4.50)
IR ν_{max}^{KBr} (cm^{-1})	3700~3000, 1700	3700~3050, 1700	3700~3050, 1700	3700~3000, 1700	3700~3050, 1700	3600~3050, 1700

Fig. 3. FAB-MS of SVD-C_1 (A) and C_2 (B).

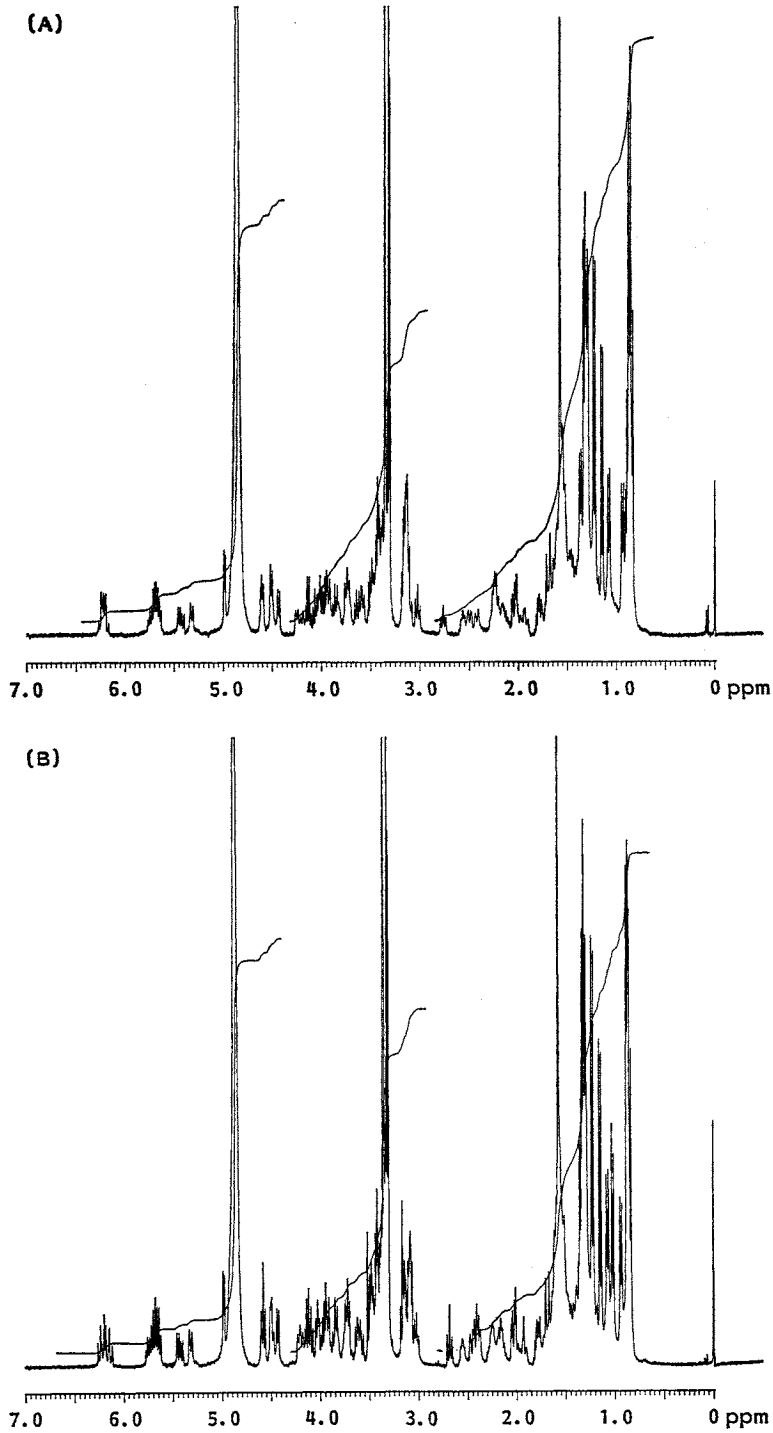


in Fig. 4 as the typical examples. They are very complicated and only the six olefinic protons at 5.4~6.3 ppm have been identified.

Biological Properties

SVD complex mixture was reported to possess an antimicrobial activity against Gram-positive bacteria, yeast and *Trichophyton*[2]. Antimicrobial activities were assayed by a conventional agar dilution method and the resulting MIC values of each component isolated in this experiment are listed in Table 2 together with that of the complex mixture. These results indicate that the antimicrobial activity of each component is essentially the same as that of the mixture. At present other biological properties of the intact SVD are being examined.

Fig. 4. ^1H NMR spectra of SVD-A_1 (A) and A_2 (B) in CD_3OD.



Interconversion of SVD

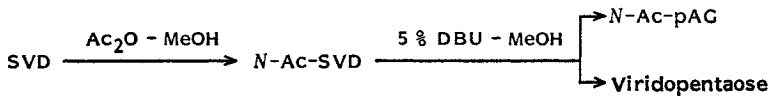
As mentioned above, we can isolate the six components of the intact SVD. To ensure the structural relationship between the free SVD and *N*-Ac-SVD, the separated components were acetylated

Table 2. Antimicrobial activity of SVD.

Test organism	MIC (μg/ml)[a]						
	SVD	A_1	A_2	B_1	B_2	C_1	C_2
Staphylococcus aureus MS 353	0.78	0.78	0.20	0.78	0.20	0.20	0.20
S. aureus MS 353 C36	0.39	0.78	0.20	0.39	0.20	0.20	0.20
S. aureus MS 353 AO	0.39	0.39	0.20	0.39	0.20	0.20	0.10
S. aureus ATCC 6538P	0.78	0.78	0.20	0.78	0.20	0.39	0.20
S. aureus 0116	1.56	1.56	0.78	0.78	0.39	0.78	0.78
S. aureus 0119	1.56	1.56	0.78	0.78	0.39	0.78	0.78
S. aureus 0126	1.56	1.56	0.78	0.78	0.78	0.78	0.78
S. aureus 0127	0.78	0.78	0.39	0.78	0.39	0.78	0.39
S. aureus Smith	1.56	0.78	0.78	0.78	0.39	0.78	0.39
S. epidermidis	1.56	1.56	0.78	1.56	0.39	0.39	0.78
Streptococcus pyogenes N.Y.5	1.56	1.56	0.78	1.56	0.78	0.78	0.78
S. pyogenes 1022	1.56	1.56	0.78	1.56	0.78	0.78	0.78
S. pyogenes S-23	1.56	1.56	0.78	1.56	0.78	0.78	0.78
S. agalactiae 1020	1.56	1.56	1.56	3.13	1.56	1.56	1.56
Enterococcus faecalis 1501	1.56	1.56	0.78	1.56	1.56	1.56	1.56
Micrococcus luteus ATCC 9341	0.78	0.78	0.20	0.78	0.39	0.39	0.39
M. flavus ATCC 10240	1.56	0.39	0.39	0.20	0.39	0.39	0.20
Corynebacterium diphtheriae PW8	1.56	0.05	0.10	0.05	0.20	0.20	0.10
Bacillus subtilis ATCC 6633	0.78	1.56	0.39	1.56	0.39	0.78	0.39
Escherichia coli NIHJ JC-2	100	>50	>50	>50	50	>50	100
Klebsiella pneumoniae ATCC 10031	>100	>50	>50	>50	100	>50	>100
Salmonella typhosa H901	100	>50	>50	>50	100	>50	>100
S. enteritidis Gärtner	>100	>50	>50	>50	>100	>50	>100
Shigella flexneri Type 3a	50	>50	50	25	25	50	50
Proteus vulgaris OX 19	50	>50	>50	>50	50	>50	100
Serratia marcescens TL-1	>100	>50	>50	>50	>100	>50	>100
Pseudomonas aeruginosa IAM 1095	>100	>50	>50	>50	>100	>50	>100

[a] Inoculum size 10^6 cells/ml.

Scheme 1. Structural relationship of SVD and related compounds.



with acetic anhydride in methanol and the resulting acetylated derivatives were identified with the standard *N*-Ac-SVD components by the reversed phase HPLC using methanol - 1 M ammonium chloride (78 : 22) as the mobile phase and FAB-MS. This supports strongly that *N*-Ac-SVD is a derivative of the intact SVD only with its amino groups being acetylated. We have already established the structural relationship between *N*-Ac-SVD and viridopentaose[5]. In this experiment, the total structural relationship including the intact SVD is now confirmed as shown in Scheme 1.

Experimental

General

MP's were determined on a micro melting point apparatus (hot-stage type, Yanagimoto MP-S3) and uncorrected. Optical rotations were measured with a Jasco DIP-181 polarimeter. IR and UV spectra were determined on a Hitachi IR-215 spectrometer and a Hitachi 200-10 double beam spectrophotometer, respectively. ^{1}H NMR spectra were measured on a Jeol GX-400 spectrometer using

TMS as an internal standard. FAB-MS were obtained on a Jeol HX-110 mass spectrometer using glycerol as the matrix. HPLC was carried out on a Shimadzu LC-3A with a Shimadzu SPD-2A spectrometer as the detector. The separation was performed on a Cosmosil 5C18 (Nacalai Tesque, 4.6×150 mm) or a Nucleosil 5C18 (Chemco, 4.6×150 mm) for analysis and a Cosmosil 5C18-P (Nacalai Tesque, 10×250 mm) for preparative separation. TLC was carried out on pre-coated plates (Kieselgel 60 F_{254}). For column chromatography, silica gel (Fuji-Davison, 150-325 mesh) and Sephadex LH-20 (Pharmacia) were used. BOND ELUT C18 (Analytical Chem) and SEP-PAK C18 (Waters) cartridges were used for desalting operations.

Separation of SVD Components

A mixture of SVD components (370 mg) was subjected to preparative HPLC to yield SVD-A_1 (7.8 mg), A_2 (13.1 mg), B_1 (12.4 mg), B_2 (10.0 mg), C_1 (21.8 mg) and C_2 (10.2 mg). Operating conditions were: column; Cosmosil 5C18-P (10×250 mm), mobile phase; MeOH - 1 M NH_4Cl (73 : 27), flow rate; 2 ml/minute, detection; UV 232 nm.

N-Acetylation of SVD Components

A solution of 1 mg of each component dissolved in 50 μl of MeOH was treated with acetic anhydride (30 μl) at room temperature. After 2 hours the reaction mixture was evaporated to dryness. The residue was analyzed by the HPLC: column; Cosmosil (4.6×150 mm), mobile phase; MeOH - 1 M NH_4Cl (78 : 22), flow rate; 1 ml/minute detection; UV 232 nm. The retention times of the six components of *N*-Ac-SVD were: A_1; 29 minutes, A_2; 17 minutes, B_1; 35 minutes, B_2; 22 minutes, C_1; 18 minutes and C_2; 13 minutes.

Acknowledgment

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