TRIACSINS, NEW INHIBITORS OF ACYL-CoA SYNTHETASE PRODUCED BY *STREPTOMYCES* SP.

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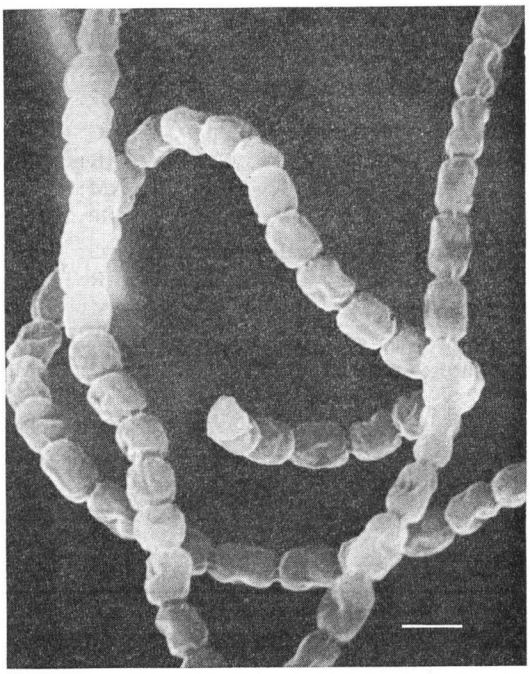
Triacsins A and B, new inhibitors of acyl-CoA synthetase, were isolated from the cultured broth of *Streptomyces* sp. strain SK-1894. The structurally related compounds WS-1228 A and B, known as hypotensive vasodilators, were also found to inhibit acyl-CoA synthetase. The four compounds have in common a *N*-hydroxytriazene moiety in their structures. The IC_{50} values for triacsin A and WS-1228 A were 5.5 and 3.6 μg/ml, respectively.

Acyl-CoA synthetase (EC 6.2.1.3) converts free fatty acids to active thioesters, acyl-CoAs. Acyl-CoA synthetase plays an important role in fatty acid metabolism and lipid biosynthesis.

During the course of our screening for new bioactive compounds from microorganisms, new inhibitors of acyl-CoA synthetase, which were named triacsins, were isolated from the cultured broth of *Streptomyces* sp. SK-1894 which was isolated from a soil sample collected at Tsurumi-ku, Kanagawa prefecture, Japan. WS-1228 A and B, reported as hypotensive vasodilators[1] and structurally related to triacsins, were also produced by SK-1894.

In this paper, the taxonomy of the producing organism, fermentation, isolation, physico-chemical properties, structure determination and biological characteristics of triacsins are described. The inhibition of acyl-CoA synthetase by WS-1228 A and B is also reported.

Plate 1. Scanning electron micrograph of spore chains of strain SK-1894 grown on inorganic salts-starch agar for 14 days.
Bar represents 1.0 μm.



Taxonomy of the Producing Strain

The International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[2] and those recommended by WAKSMAN[3] were used. Morphological, cultural and physiological characteristics were observed after incubation at 27°C for two weeks. The utilization of carbon sources was tested by growth at 27°C on PRIDHAM and GOTTLIEB's medium containing 1% of each carbon source. The chemical analysis of 2,4-diaminopimelic acid (A_2pm) in the cell wall was

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Table 1. Cultural characteristics of strain SK-1894.

Yeast extract - malt extract agar*	G:	Good, light amber (3ic)
	R:	Light amber (3ic)
	AM:	Abundant, powdery, parchment (1-db)
	SP:	Bamboo (2gc)
Oatmeal agar*	G:	Moderate, bamboo (2gc)
	R:	Light wheat (2ea)
	AM:	Moderate, powdery, natural (3dc) or light ivory (2ca)
	SP:	Bamboo (2gc)
Inorganic salts - starch agar*	G:	Good, topaz (3ne)
	R:	Light amber (3ic)
	AM:	Abundant, powder, ashes (5fe) or light wheat (2ea)
	SP:	None
Glycerol - asparagine agar*	G:	Moderate, yellow maple (3le)
	R:	Sand (3cb) or yellow maple (3le)
	AM:	Very poor, white (a)
	SP:	None
Glucose - asparagine agar	G:	Poor, colorless or yellow maple (3le)
	R:	Pearl pink (2ca)
	AM:	Very poor, white (a)
	SP:	None
Peptone - yeast extract - iron agar*	G:	Good, center; yellow maple (3le), outside; pearl pink (3ca)
	R:	Yellow maple (3le)
	AM:	None
	SP:	None
Tyrosine agar*	G:	Moderate, camel (3ie)
	R:	Camel (3ie)
	AM:	Very poor, white (a)
	SP:	None
Sucrose - nitrate agar*	G:	Very poor, colorless
	R:	White (a)
	AM:	Very poor, white (a)
	SP:	None
Glucose - nitrate agar**	G:	Poor, light ivory (2ca) or light amber (3ic)
	R:	Light ivory (2ca) or light amber (3ic)
	AM:	Very poor, white (a)
	SP:	None
Glycerol - calcium malate agar**	G:	Good, light amber (3ic)
	R:	Light amber (3ic)
	AM:	Very poor, white (a)
	SP:	None
Glucose - peptone agar**	G:	Moderate, yellow maple (3le)
	R:	Yellow maple (3lc)
	AM:	Poor, white (a)
	SP:	None
Nutrient agar**	G:	Good, dark luggage tan (4pg)
	R:	Dark luggage tan (4pg)
	AM:	Very poor, white (a)
	SP:	Poor, bamboo (2fb)

* Medium recommended by ISP.

** Medium recommended by S.A. WAKSMAN.

Abbreviations: G; Growth of vegetative mycelium, R; reverse, AM; aerial mycelium, SP; soluble pigment.

Table 2. Physiological properties of strain SK-1894.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Liquefaction of gelatin (21°C)	+
Peptonization of milk (37°C)	+
Coagulation of milk (37°C)	−
Cellulolytic activity	−
Hydrolysis of starch	−
Temperature range for growth	15~42°C

+: Active, −: not active.

Table 3. Utilization of carbon sources by strain SK-1894.

D-Glucose	+
D-Fructose	±
L-Rhamnose	−
D-Mannitol	−
L-Arabinose	−
i-Inositol	+
Raffinose	−
D-Xylose	+
Sucrose	±
Melibiose	−

+: Utilized, ±: weakly utilized, −: not utilized.

carried out by the method of LECHEVALIER and LECHEVALIER[4].

Morphology

Moderate or good growth of aerial mycelia in strain SK-1894 were observed on yeast extract - malt extract agar, oatmeal agar and inorganic salts - starch agar. The sporophores were terminated by long chains of more than 20 spores which form straight or open loops. Therefore, spore chains of this strain belongs to the type *Rectiflexibiles* or *Retinaculiaperti*.

The spores are cylindrical in shape, $0.5 \times 0.7 \sim 0.9$ μm in size, and have a smooth surface (Plate 1). The vegetative mycelia of strain SK-1894 do not show fragmentation into coccoid or bacillary elements. Sclerotic granules, sporangia and flagellated spores were not observed.

Chemical Composition

The cell wall of the strain SK-1894 contained LL-A_2pm.

Cultural and Physiological Characteristics

The cultural and physiological characteristics, and the utilization of carbon sources of strain SK-1894 are shown in Tables 1, 2 and 3, respectively. Color names and hue numbers indicated in Table 1 are those of Color Harmony Manual (4th Ed.) published by Container Corporation of America.

The yellowish brown vegetative mycelia of strain SK-1894 grew abundantly on organic agar media. Color of aerial mycelia was gray or white.

Based on these results, strain SK-1894 is considered to belong to the genus *Streptomyces* and to be a strain of the gray series of the PRIDHAM and TRESNER grouping[5].

Strain SK-1894 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. SK-1894 with the accession No. FERM P-8655.

Assay Method

NEFA *C*-test kit (Wako Pure Chemical Industries, Ltd.) was used to assay the activity of acyl-CoA synthetase. The details of the method were described in the Biological Properties section.

Fermentation and Isolation

Spores and vegetative mycelia of strain SK-1894 was inoculated into 100 ml of a seed medium (pH 7.0) consisting of glucose 0.1%, starch 2.4%, peptone 0.3%, meat extract 0.3%, yeast extract 0.5% and $CaCO_3$ 0.4% in a 500-ml Erlenmeyer flask, which was incubated on a rotary shaker at 27°C for 2 days. Two hundred milliliters of the seed culture were transferred to 20 liters of a production medium (pH 6.5) containing glucose 1.0%, soluble starch 1.0%, corn steep liquor 0.3%, oatmeal 1.0%, Parmamedia

Fig. 1. HPLC chromatogram of a crude sample of triacsins.

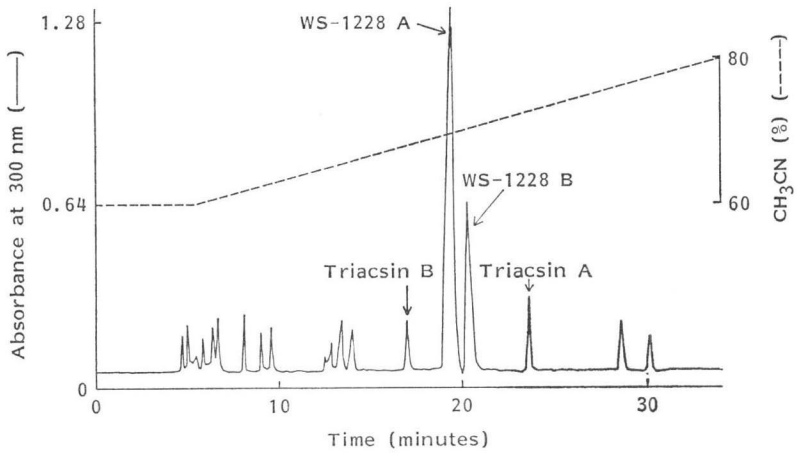
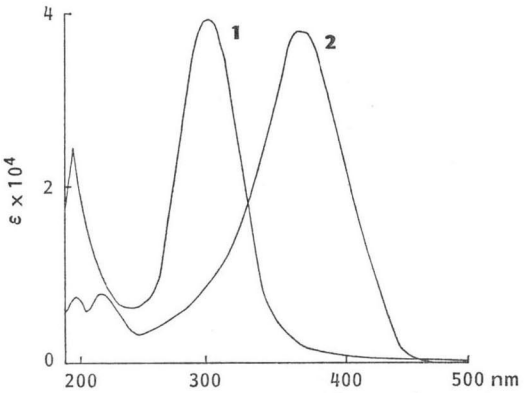


Table 4. Physico-chemical properties of triacsins A and B.

	Triacsin A	Triacsin B
Nature	Yellow powder	Yellow powder
MP (°C)	116~118	144~147
Formula	$C_{11}H_{19}N_3O$	$C_{11}H_{15}N_3O$
HR-MS Calcd	209.1527	205.1214
Found	209.1537	205.1213
UV $\lambda_{max}^{70\%CH_3CN}$ nm (ε)	300 (40,000)	363 (39,000)
^{1}H NMR (δ ppm)	8.36 (d, H, J=10.0 Hz), 7.02 (dd, H, J=15.0, 10.5 Hz), 6.39 (dd, H, J=15.0, 10.0 Hz), 6.35 (dd, H, J=15.2, 11.0 Hz), 6.20 (dt, H, J=15.2, 7.0 Hz), 2.21 (dt, H, J=7.0, 7.0 Hz), 1.45 (m, 2H), 1.35~1.32 (m, 6H), 0.9 (t, 3H, J=7.0 Hz)	8.38 (d, H, J=10.0 Hz), 7.08 (dd, H, J=15.0, 11.2 Hz), 6.67 (dd, H, J=15.0, 10.5 Hz), 6.48~6.45 (m, 3H), 6.29 (dd, H, J=15.0, 10.5 Hz), 6.18 (dd, H, J=15.3, 10.5 Hz), 5.92 (dt, H, J=15.3, 6.8 Hz), 2.16 (m, 2H), 1.03 (t, 3H, J=7.5 Hz)
^{13}C NMR (δ ppm)	170.1 (d), 149.9 (d), 146.0 (d), 131.3 (d), 126.3 (d), 34.4 (t), 33.1 (t), 30.3 (t), 30.2 (t), 24.0 (t), 14.7 (q)	169.6 (d), 149.3 (d), 142.6 (d), 141.4 (d), 139.7 (d), 131.9 (d), 131.6 (d), 131.2 (d), 127.4 (d), 27.3 (t), 14.1 (q)

1.0% and basic magnesium carbonate 0.5% in a 30-liter jar fermentor. The fermentation was carried out at 27°C with aeration of 10 liters/minute and agitation of 250 rpm. Acyl-CoA synthetase inhibiting activity of the cultured broth appeared at 20 hours after the inoculation, then gradually increased and reached a maximum at 60 hours. The cultured broth (15 liters) was centrifuged to obtain about 12 liters of a supernatant fluid. The mycelial cake was extracted with 1.5 liters of 80% aqueous acetone. After the removal of acetone by evaporation under reduced pressure, the aqueous solution was combined with the supernatant fluid. The mixture was extracted with 13 liters of ethyl acetate. The extracts were concentrated *in vacuo* to dryness to yield a brown paste (5.5 g). The paste, dissolved in 30 ml of chloroform, was applied to a silica gel column (E. Merck, Kieselgel 60, 250 g), and then the active components were eluted with chloroform - methanol (75 : 1). The active fractions were concentrated *in vacuo* to give a brown powder (600 mg). The powder was finally purified by HPLC (Jasco Tri Rotar V system,

Fig. 2. UV spectra of triacsins A (1) and B (2) in 70% aq CH_3CN.



column: YMC Pack A-343 ODS, 20×250 mm. Solvent: A 30-minute linear gradient from 60% to 80% aqueous CH_3CN. Flow rate: 8.0 ml/minute. Detection: UV at 300 nm). As shown in Fig. 1, a typical chromatographic run yielded four active fractions: Fractions 1, 2, 3 and 4 with retention times at 16.9, 19.5, 20.3 and 23.6 minutes, respectively. Triacsin A (7 mg) was obtained from fraction 4 and triacsin B (5 mg) from fraction 1. Compounds from fractions 2 (50 mg) and 3 (30 mg) were identified as WS-1228 A and B[1,6], respectively.

Physico-chemical Properties

The physico-chemical properties of triacsins A and B are summarized in Table 4. They are soluble in methanol, ethanol, acetonitrile, acetone, ethyl acetate, slightly soluble in chloroform and benzene and insoluble in water. The UV spectrum (Fig. 2) shows a maximum at 300 nm (ε 40,000) for triacsin A and a maximum at 363 nm (ε 39,000) for triacsin B. The IR spectrum ($CHCl_3$) of triacsin A is shown in Fig. 3.

Structure Determination

The physico-chemical properties of triacsin A are closely similar to those of WS-1228 A[1,6]. The molecular formular of triacsin A was determined to be $C_{11}H_{10}N_3O$ by high resolution mass spectrometry (Table 4) which was 2 mass units larger than that of WS-1228 A. The IR (1570, 1608 cm^{-1}, Fig. 3) and UV (363 nm, Fig. 2) spectra suggested that triacsin A conserved the triene oxime moiety[6]. From these data, triacsin A appears to be the 7,8-dihydro derivative of WS-1228 A. In fact, the ^1H NMR spectrum

Fig. 3. IR spectrum of triacsin A ($CHCl_3$).

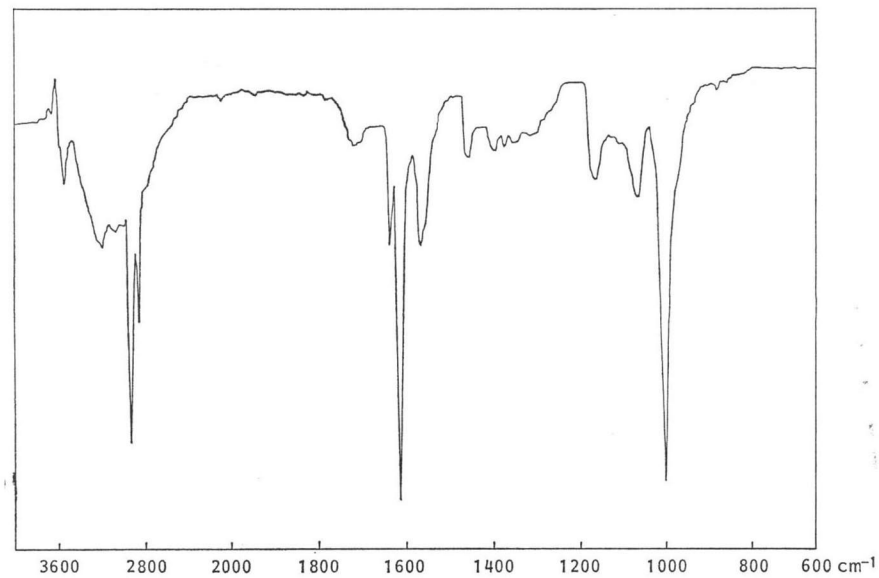


Fig. 4. ^{13}C NMR spectra of triacsins A and B (100 MHz in CD_3OD).
* Two signals (30.2 and 30.3 ppm).

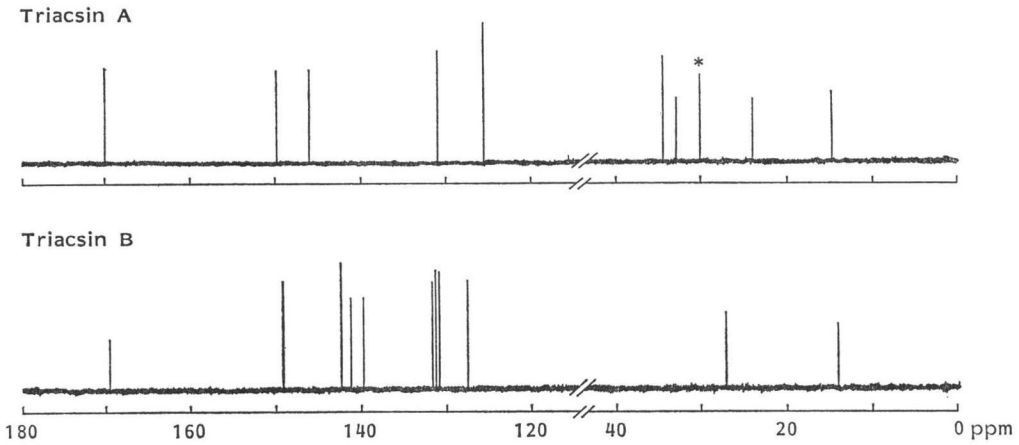
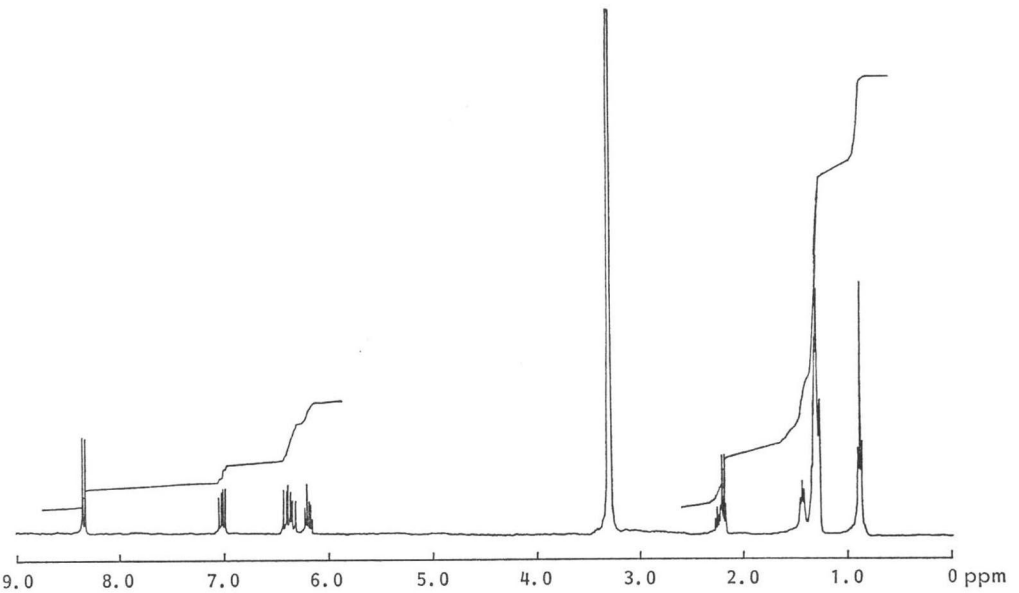


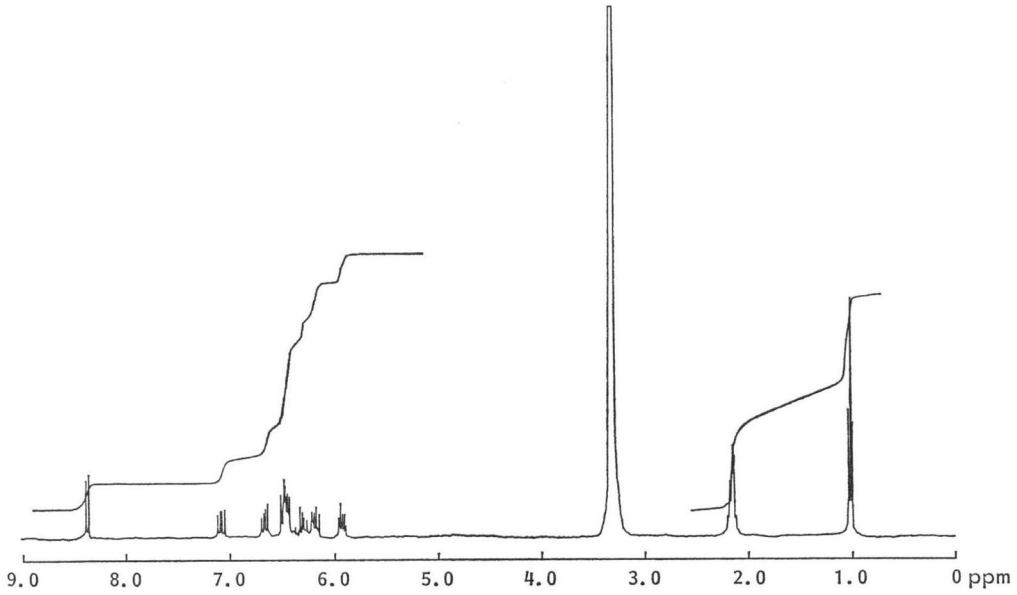
Fig. 5. ^1H NMR spectrum of triacsin A (400 MHz in CD_3OD).



of triacsin A in CD_3OD (Fig. 5) shows the signals summarized in Table 4 which are assignable to *E,E*-2,4-undecadienylidine moiety. This structure was also confirmed by ^{13}C NMR spectrum (Fig. 4 and Table 4). Based on the above data, the structure of triacsin A was determined as **1**.

The UV spectrum of triacsin B (363 nm, Fig. 2) showed the red wave shift (24 nm) in comparison with that of WS-1228 B (339 nm)[1]. From the UV shift and IR spectrum (1570, 1608 cm^{-1}, data not shown) of triacsin B, it was suggested to have the structure of hexaene oxime moiety. The molecular formula of triacsin B was also determined to be $C_{11}H_{15}N_3O$ which was 2 mass units smaller than that of WS-1228 B. The ^{13}C NMR (Fig. 4 and Table 4) and ^1H NMR (Fig. 6 and Table 4) spectra suggest structure **2** for triacsin B. It is the 8,9-dehydro derivative of WS-1228 B.

Fig. 6. ^1H NMR spectrum of triacsin B (400 MHz in CD_3OD).



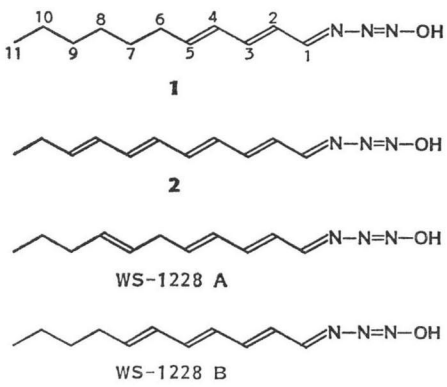
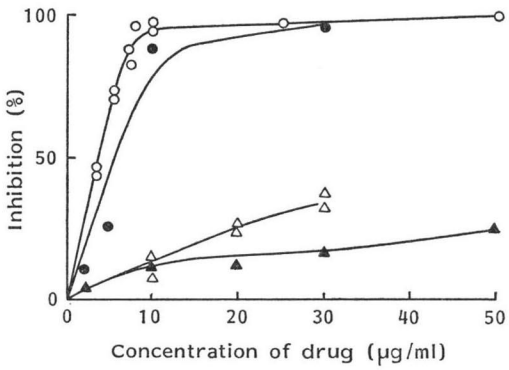


Fig. 7. Effect of triacsins A (●), B (▲), WS-1228 A (○) and B (△) on acyl-CoA synthetase.



Biological Properties

Triacsins A and B showed no antimicrobial activity at the concentration of 100 μg/ml.

NEFA *C*-test kit (Wako Pure Chemical Industries, Ltd.) was used to assay the activity of acyl-CoA synthetase according to the method described in the manual of the kit. The acyl-CoA synthetase used in the kit is purified from a bacterium (*Pseudomonas*?). Ten microliters of inhibitors was added to 1 ml of solution A (containing acyl-CoA synthetase) and preincubated for 10 minutes at 37°C. The reaction was initiated by the addition of 50 μl of oleic acid (1 mEq/liter). After incubation for 10 minutes at 37°C, 2 ml of solution B was added to the mixture. The resulting mixture was incubated for 10 minutes at 37°C. The absorbance was measured at 550 nm in a cuvette of 1-cm light path with a Shimadzu UV-240 spectrophotometer. Fig. 7 shows that triacsins A, B, WS-1228 A and B inhibit acyl-CoA synthetase. Among them triacsin A and WS-1228 A are more potent with IC_{50} values of 5.5 and 3.6 μg/ml, respectively.

Acyl-CoA synthetase partially purified from rat liver was also inhibited. IC_{50} values for triacsin A and WS-1228 A were 5.0 and 1.0 μg/ml, respectively.

Discussion

The present investigation shows that triacsins and WS-1228s inhibit acyl-CoA synthetase. Of the four compounds triacsin A and WS-1228 A are more potent (Fig. 7). They have in common a *N*-hydroxytriazene moiety which appears to be responsible for inhibitory activity.

WS-1228 A and B were reported as hypotensive vasodilators[1,6]. WS-1228 B is a much more potent hypotensive vasodilator than WS-1228 A[1]. On the contrary, the inhibitory activity against acyl-CoA synthetase of WS-1228 A is more than 10 times greater than that of WS-1228 B (Fig. 7). From these observation there might be no relationship between the two activities, though any other definite evidence is not presented. However, it will be interesting to see if triacsins show hypotensive activity. Acute toxicity of triacsins has not been tested but they would be expected to show low toxicity since the acute toxicity of WS-1228 B was reported to be about 250 mg/kg (ip mice)[1].

Further evaluation of the biological properties of triacsins are in progress. The screening method of acyl-CoA synthetase inhibitors will be published in near future.

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