Sattabacins and Sattazolins: New Biologically Active Compounds with Antiviral Properties Extracted from a *Bacillus* sp.

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A series of new compounds with antiviral properties were isolated from a *Bacillus* sp. strain B-60. They were named sattabacin (**1**), hydroxysattabacin (**2**), sattazolin (**3**) and methylsattazolin (**4**). The biologically active compounds were recovered from fermentation broth by ethyl acetate extraction and silica-gel column fractionation. Their antiviral activity was mainly expressed against the Herpes simplex viruses type 1 and 2. The compound **3** showed a selective inhibition of protein synthesis in Herpesvirus-infected cells.

Bacteria have been shown to be a common source of antibiotics and other biologically active substances[1~4]. In addition, it has been claimed that original antiviral drugs are produced by some species of the genus *Bacillus*[5,6].

During a screening program for the detection of biologically active compounds from environmental microorganisms, we isolated and selected a *Bacillus* sp. which produced two types of novel compounds, namely sattabacins and sattazolins, endowed with specific antiviral properties.

Taxonomy of Producing Strain

A strain of aerobic, spore-forming bacillus was isolated from soil samples taken in Cagliari (Sardinia, Italy). It was identified as a species close to *Bacillus lentus* and *B. firmus*, through its morphological, biochemical and physiological properties and also by using the API 50 CHB system (Bio-Mériéux, Italia), but, it differed from the former in arbutin, gelatin and citrate tests, and from the latter in glycerol, arbutin and urease tests (Table 1). This strain was designated as *Bacillus* sp. B-60 and deposited in Czech Collection of Microorganisms with the No. CCM 4466.

Production

The *Bacillus* B-60 was grown in an Erlenmeyer flask on the medium AR-2, which had the following composition: yeast extract 2 g, casaminoacids 10 g, soluble starch 4 g, NaCl 6 g, KCl 0.4 g, $CaCl_2 \cdot 2H_2O$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g in 1 liter of deionized water. The final pH was adjusted to 7.2.

After 18 hours at 28°C under shaking conditions (250 rpm) in a dry incubator (New Brunswick model G25), the microbial suspension was seeded in agar plates of medium AR-2 with the addition of 11 g/liter of purified agar (Oxoid, Basingstoke, U.K.). These were incubated for 5 days at 28°C. Subsequently the plates underwent a cycle of freezing and thawing to extract the medium together with the products of fermentation. The extract was clarified by centrifuging at 8000 rpm for 15 minutes.

Isolation

Scheme 1 summarises the steps followed to obtain the pure substances. The solution (30 liters) was extracted twice with 20% EtOAc (v/v). The solvent was collected and dried in a vacuum evaporator. The crude extract (1.3 g) was chromatographed on a silica gel column eluted with an increasing amount of EtOAc in C_6H_6 affording

Table 1. Physiological and biochemical proprierties of the *Bacillus* B-60 strain.

Test performed	Result	Test performed	Result
Arginine	−	Tryptophane	−
Lysine	−	Indole production	−
Ornithine	−	Sodium pyruvate	−
Sodium citrate	−	Gelatinase	−
Sodium thiosulphate	−	Oxidase	−
Urease	−	NO_2 production	+
Yellow pigment production	+	N_2 production	+
Growth on McConkey	−	Growth on 2% NaCl	+
Growth on Blood-Agar	+	Growth on 5% NaCl	−
Erythritol	−	D-Mannose	−
D-Arabinose	−	L-Sorbose	−
L-Arabinose	−	L-Rhamnose	−
Ribose	−	Inositol	−
D-Xylose	−	D-Mannitol	−
L-Xylose	−	D-Sorbitol	−
Adonitol	−	1-o-Methyl α-D-mannoside	−
1-o-Methyl β-D-xyloside	−	1-o-Methyl α-D-glucoside	−
D-Galactose	−	n-Acetylglucosamine	+
D-Glucose	+	Amygdalin	−
D-Fructose	−	Arbutin	+
Esculin	+	Melezitose	−
Salicin	−	Raffinose	−
D-Cellobiose	−	Soluble starch	+
D-Maltose	+	Glycogen	−
Melibiose	−	Xylitol	−
Sucrose	−	Gentiobiose	−
Trehalose	+	D-Turanose	−
Inulin	−	D-Lyxose	−
D-Tagatose	−	2-Ketogluconate	−
L-Fucose	−	5-Ketogluconate	−
D-Fucose	−	Glycerol	+
L-Arabitol	−	o-Nitrophenylgalactoside	+
D-Arabitol	−	Dulcitol	−
Lactose	−	Gluconate	−

Scheme 1. Purification steps for sattabacins and sattazolins.

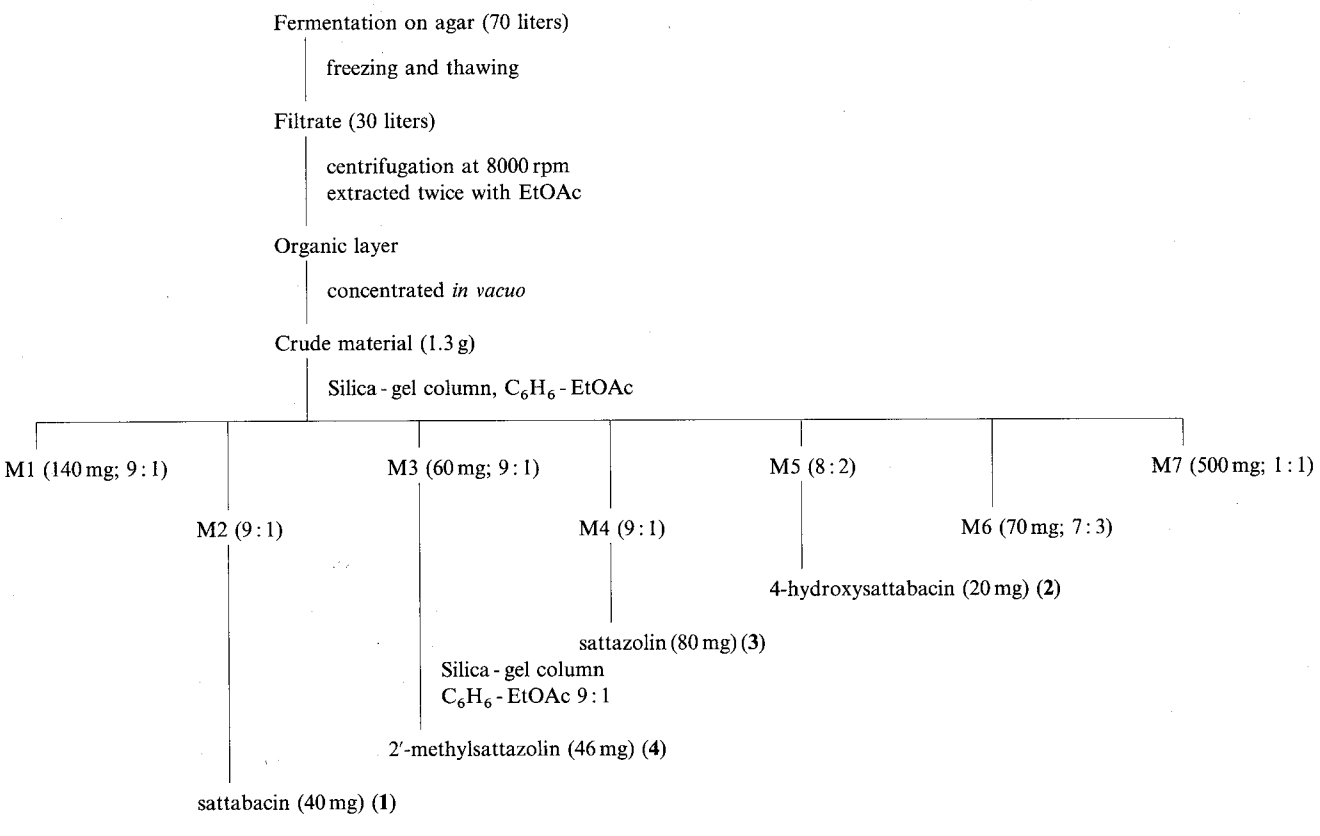
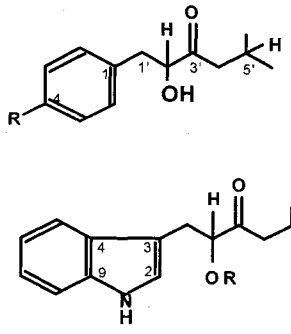


Fig. 1. Structure of sattabacins and sattazolins.



	R	
1	H	sattabacin
2	OH	4-hydroxysattabacin

	R	
3	H	sattazolin
4	Me	2'-methylsattazolin

the fractions M1 (140 mg; C_6H_6 - EtOAc, 9 : 1 (v/v)), M2 (40 mg; 9 : 1), M3 (60 mg; 9 : 1), M4 (80 mg; 9 : 1), M5 (20 mg; 8 : 2), M6 (70 mg; 7 : 3) and M7 (500 mg; 1 : 1). M2, M4 and M5 were pure compounds and were named, respectively, sattabacin (**1**), sattazolin (**3**) and 4-hydroxysattabacin (**2**).

Further purification of M3 (Silica gel; C_6H_6 - EtOAc, 9 : 1) gave 2'-methylsattazolin (**4**; 46 mg). The formulae of these compounds are given in Figure 1.

Structure Elucidation and Physico-chemical Properties of Compounds

Sattabacin (**1**) and 4-hydroxysattabacin (**2**): Compound **1**, $C_{13}H_{18}O_2$, exhibited the tropilium ion (*m/z* 91) in the mass spectrum and NMR signals for a mono-substituted benzene. In addition, the ^{13}C/APT spectra (Table 2) showed resonances for two methylenes, one saturated carbonyl, two methyls and two methines (one of which oxygenated), for the $C_7H_{13}O_2$ chain. Multiplicities of the ^1H NMR signals and decoupling experiments revealed the presence of two separated partial structures for the chain, respectively, $-CH_2-$ CH(OH)- and $-CH_2-CH(Me)_2$. These data may be arranged as in structure **1**. Accordingly, **1** exhibited ions at *m/z* 149 and *m/z* 121 in the mass spectrum, due to the losses of C_4H_9 and C_5H_9O fragments, respectively, from the molecular ion.

Compound **2**, $C_{13}H_{18}O_3$, was assigned the structure **2** because it showed the same NMR signals for the chain of **1** and other signals attributable to a p-hydroxybenzene.

Sattazolin (**3**) and 2'-methylsattazolin (**4**): Compound **3**, $C_{15}H_{19}NO_2$, again exhibited the same NMR data as **1** for the chain (Table 2). Notably, the ^{13}C NMR signal attributed to the methylene in position 2' was shielded (10 ppm) indicating a different aromatic moiety to which the $C_7H_{13}O_2$ chain is linked. With respect to the C_8H_6N aromatic part, it gave ^1H and ^{13}C/APT signals for five methines, three quaternary carbons and one NH (δ 8.15 in the ^1H NMR spectrum), which may be arranged as a mono-substituted indole nucleus. Comparison of the ^{13}C NMR data (Table 2) with those of monomethyl indoles[7,8] established the location of the chain on C-3.

Table 2. ^{13}C NMR data for **1**~**4** ($CDCl_3$; 75 MHz).

C	1	2	3	4
1	136.6	128.1	—	—
2	129.2	130.4	122.9	122.9
3	128.5	115.4	110.5	111.1
4	126.8	154.8	127.4	127.4
5	128.5	115.4	118.6	118.7
6	129.2	130.4	119.5	119.4
7	—	—	122.1	122.0
8	—	—	111.2	111.1
9	—	—	136.1	136.0
1'	40.0	39.1	29.7	27.8
2'	77.4	77.6	76.9	87.2
3'	211.2	211.4	211.8	212.3
4'	47.4	47.4	47.3	47.3
5'	24.5	24.6	24.4	23.6
6'	22.6	22.6	22.5	22.6
7'	22.2	22.5	22.5	22.6
OMe	—	—	—	58.2

The only major difference between the NMR spectra of compound **3** and **4**, $C_{16}H_{11}NO_2$, was the presence in the latter of a signal more commonly (δ_H 3.34 and δ_C 58.2) attributed to a methoxy group. The location of this substituent on C-2' was indicated by the deshielding (δ 87.2 *vs.* δ 76.9) of this signal in the ^{13}C NMR spectrum (Table 2).

Biological Properties

Cytotoxicity

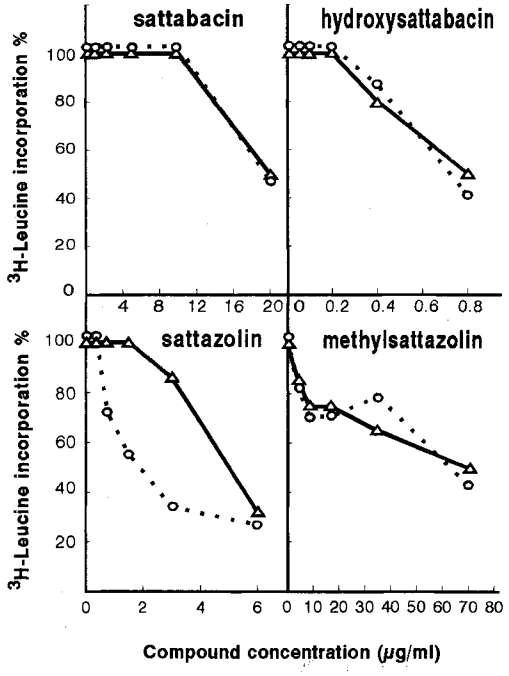
The toxicity of the new compounds, evaluated on VERO cell monolayers as the incorporation of [^3H]-leucine is indicated in Table 3. The minimal toxic dose 50% (MTD_{50}) was the concentration of the compounds which inhibited by 50%, the [^3H]-leucine uptake by cells.

Table 3. Cytotoxicity and antiviral activity of sattabacins and sattazolins.

Compound	[^{3}H]-leucine uptake MTD_{50} (μg/ml)	Inhibition of virus plaque production ID_{50} (μg/ml)			
		HSV1	HSV2	VV	1S
Sattabacin (**1**)	20	3	3	7.5	7.5
Hydroxysattabacin (**2**)	0.8	0.32	0.08	0.8	0.8
Sattazolin (**3**)	4.5	1.5	1.5	4.5	4.5
Methylsattazolin (**4**)	70	35	>70	50	50

MTD_{50}=Minimal Toxic Dose 50%.

Fig. 2. Incorporation of [^{3}H]-leucine in either uninfected (-△-) or HSV2 infected (-○-) VERO cells in the presence of sattabacins and sattazolins.



The drugs were added at the third hour post-infection. Then 1 μCi/ml of [^{3}H]-leucine was added at the 4th hour post-injection and left for a further hour.

MTD_{50} was 0.8 μg/ml for **2**, 4.5 μg/ml for **3**, 20 μg/ml for **1** and 70 μg/ml for **4**. At these concentrations, no alterations of [^{3}H]-uridine or [^{3}H]-thymidine uptake were observed (data not shown). Overlapping results were obtained with culture cell counts (data not shown).

Antiviral Activity

Herpes simplex virus type 1 (HSV1), Herpes simplex virus type 2 (HSV2), Poliovirus type 1 (1S) and Vaccinia virus (VV) were screened.

The anti-viral activity of the new compounds, tested with a plaque reduction assay is indicated in Table 3 and is refered as ID_{50}.

In the virus plaque reduction assay, the ID_{50} for 1 was 3 μg/ml on both HSV1 and HSV2, 1.5 μg/ml for **3**, 35 μg/ml for compound **4** on HSV1 and as low as 0.08 μg/ml for **2** on HSV2; this last compound was a little less active on HSV1 (0.3 μg/ml). Almost similar results were obtained with the virus yield reduction assay (data not shown). All the compounds showed a virustatic activity.

The compounds did not show any evident antibiotic activity on *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*.

Macromolecular Precursor Uptake

The incorporation of [^{3}H]-leucine in both virus-infected and uninfected cells is shown in Fig. 2. Compound **1** at 20 μg/ml inhibited the incorporation of [^{3}H]-leucine in HSV2-infected and uninfected cells by 50%. **3** at the dose of 1.5 μg/ml inhibited the uptake of [^{3}H]-leucine in virus-infected cells by 45%, but did not do so in uninfected cells.

Compound **2** at 0.8 μg/ml inhibited the uptake of [^{3}H]-leucine in both the uninfected and infected cells by about 50%. Compound **4** at 20 μg/ml inhibited the uptake of [^{3}H]-leucine in both the uninfected and infected cells by 50%.

Discussion

Compounds **1** and **2** showed quite different potency, in spite of their very close chemical structure. The hydroxyl group in position 4 made the last compound much more active on viruses (but its cytotoxicity was also enhanced).

2 was the most active anti-herpetic compound with a protection index of about 10 on HSV2 and about 3 on HSV1.

Compound **3** was less active than **1** and **2** on viruses. The methylation in position 2′ induced a great decrease of the toxic activity of compound **4**, but also of its antiviral activity, especially on HSV2.

The mechanism of action of these new compounds has

not yet been clarified. One of the compounds (**3**) seems to affect protein synthesis at the highest doses used, with a selectivity between infected and non-infected cells.

Further work is needed to elucidate the structure-activity relationship, with the aim of modifying these new compounds or of synthesizing other more selective derivatives.

Experimental

NMR spectra were obtained on a Varian Gemini-300 spectrometer in $CDCl_3$ (TMS as reference). Mass spectra were run on a AEI 12 instrument at 70 eV.

Sattabacin (**1**)

$C_{13}H_{18}O_2$. $[\alpha]_D = +35°$ (*c* 0.7, $CHCl_3$). ^1H NMR (300 MHz): δ 7.32~7.20 (m, C_6H_6), 4.36 (dd, J=7.4 and 4.4 Hz; H-2′), 3.47 (broad s; OH), 3.12 (dd, J=14.2 and 4.4 Hz; H-1′a), 2.81 (dd, J=14.2 and 7.7 Hz; H-1′b), 2.36 (d, J=6.8 Hz; H_2-4′); 2.17 (eptet; H-5′), 0.91 (d, J=6.7 Hz; Me-6′, Me-7′). ^{13}C in Table 2. HREI-MS calcd for $C_{13}H_{18}O_2$: 206.1307, found 206.1310. EI-MS, *m/z* (rel. int.): 206 (37) $[M]^+$, 188 (35), 149 (47) $[M-C_4H_9]^+$, 121 (70) $[M-C_5H_9O]^+$, 91 (100).

4-Hydroxysattabacin (**2**)

$C_{13}H_{18}O_3$. $[\alpha]_D = +14°$ (*c* 0.3, $CHCl_3$). ^1H NMR (300 MHz); δ 7.07 (d, J=8.3 Hz; H-2, H-6), 6.73 (d, J=8.3 Hz; H-3, H-5), 4.34 (dd, J=7.4 and 4.4 Hz; H-2′), 3.06 (dd, J=14.3 and 4.4 Hz; Hz; H-1′a), 2.75 (dd, J=14.3 and 7.4 Hz; H-1′b), 2.37 (d, J=6.6 Hz; H_2-4′), 2.18 (eptet; H-5′), 0.92 (d, J=6.6 Hz; Me-6′, Me-7′). ^{13}C in Table 2. HREI-MS calcd for $C_{13}H_{18}O_3$: 222.1256, found 222.1260.

Sattazolin (**3**)

$C_{15}H_{19}NO_2$. $[\alpha]_D = +58°$ (*c* 1.5, $CHCl_3$). ^1H NMR (300 MHz); δ 8.15 (broad s; NH), 7.62 (d, J=7.7 Hz; H-8), 7.32 (d, J=7.7 Hz; H-5), 7.19 (t, J=7.7 Hz; H-6), 7.12 (t, J=7.7 Hz; H-7), 7.05 (d, J=2.4 Hz; H-2), 4.45 (broad t, J=7 Hz; H-2′), 3.52 (broad s; OH), 3.20 (dd, J=15 and 4.5 Hz; H-1′a), 3.05 (dd, J=15 and 7 Hz; H-1′b), 2.38 (d, J=7 Hz; H_2-4′), 2.15 (eptet; H-5′), 0.89 and 0.86 (d, J=6.6 Hz; Me-6′, Me-7′). ^{13}C NMR in Table 2. HREI-MS calcd for $C_{15}H_{19}NO_2$: 245.1416, found 245.1415. EI-MS, *m/z* (rel. int.): 245 (45) $[M]^+$, 130 (100).

2′-Methylsattazolin (**4**)

$C_{16}H_{21}NO_2$. $[\alpha]_D = -22°$ (*c* 0.5, $CHCl_3$). ^1H NMR (300 MHz); δ 8.10 (broad s; NH), 7.63 (d, J=7.7 Hz; H-8), 7.34 (d, J=7.7 Hz; H-5), 7.19 (t, J=7.7 Hz; H-6), 7.12 (t, J=7.7 Hz; H-7), 7.04 (d, J=2.4 Hz; H-2), 3.92 (dd, J=7.2 and 5.2 Hz; H-2′), 3.34 (s; OMe), 3.12 (dd, J=15 and 5.2 Hz; H-1′a), 3.07 (dd, J=15 and 7.2 Hz; H-1′b), 2.36 (d, J=7.5 Hz; H_2-4′), 2.13 (eptet; H-5′), 0.87 and 0.84 (d, J=6.6 Hz; Me-6′, Me-7′). ^{13}C in Table 2. HREI-MS calcd for $C_{16}H_{21}NO_2$: 259.1572, found 259.1570.

Cell and Viruses

Vero cells, purchased from ICN-Flow (Costa Mesa, Ca) were used for cytotoxicity experiments and for the antiherpes assays. The cells were grown in Dulbecco's modified Eagle medium with 10% fetal calf serum.

HSV1, HSV2, 1S and VV were obtained from NIH (Bethesda, Md).

Bacterial and Fungal Strains

For antibacterial and antifungal assays the following strains were used: *Staphylococcus aureus* ATCC 25932, *Enterococcus faecalis* ATCC 27989, *Escherichia coli* ATCC 25922. *Candida albicans* PR-1 was from our Institute's collection and was identified according to conventional procedures[9].

Cytotoxicity and Antiviral Activity Assays

The cytotoxicity of the biologically active extracts was evaluated by inhibition tests of cell multiplication according to SERRA *et al.*[10] and by inhibition of radioactive precursor incorporation into cellular macromolecules. [^{3}H]-leucine, [^{3}H]-uridine and [^{3}H]-thymidine were used as indicators of cell macromolecular biosynthesis. All the experiments were done in triplicate.

Antiviral activity of the compounds was evaluated both with a plaque reduction test and with a virus yield assay. The plaque reduction test was performed according to SERRA *et al.*[10]. The viral yield reduction assay was evaluated on cell monolayers of VERO cells according to HAYASHI *et al.*[11].

For testing the effect of the new compounds on the macromolecular synthesis in infected cells, cell monolayers which had either been infected or mock infected with HSV1 and HSV2, were incubated with the drugs for variable times; then [^{3}H]-leucine was added for one hour. After this time, the cells were washed, detached from the plates and the radioactivity was counted in a β-counter.

Antibiotic Activity Assay

Antibacterial and antifungal activity was studied by a dilution method in multiwell plates using Muller-Hinton broth (for bacteria)[12] and Sabouraud broth (for fungi), according to standard conditions[13].

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