Preparation of 13-*epi*-Selamectin by Biotransformation Using a Blocked Mutant of *Streptomyces avermitilis*

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(Received for publication August 3, 1999)

Selamectin[1], (5*Z*,13*S*)-α-oleandrosyl-25-cyclohexyl-25-de(1-methylpropyl)-5-deoxy-22,23-dihydro-5-(hydroxyimino)-avermectin B1 monosaccharide is active against fleas and ticks, intestinal hookworms and roundworms, and immature heartworm[1] and has been commercialized as Revolution® (U.S.A) and Stronghold® (Europe), an endectocide for companion animals. We describe here the preparation, through biotransformation, of 13-*epi*-selamectin using a blocked mutant of *S. avermitilis*, 15-63, as part of our investigation into the SAR of flea activity with this class of compounds.

A loopful of the *S. avermitilis* culture 15-63 which is stored on 1/4 strength ATCC172 agar was inoculated into two 300 ml Erlenmeyer shake-flasks containing 50 ml of AS-7 inoculum medium comprising Nadex® starch 2.0%, Pharmamedia® cotton seed meal 1.5%, Ardamine® nitrogen source 0.5% and $CaCO_3$ 0.2% adjusted to pH 7.2 with 1 M NaOH solution. After 48 hours incubation at 28°C on a rotary shaker at 200 rpm, these flasks were used to inoculate 3.5 litres of AP-5 medium in a 5 litre mini-jar (Electrolab, Gloucester, U.K. GL20 7LR). AP-5 medium consists of Nadex® starch 8%, Oxoid® yeast extract 0.5%, $MgSO_4 \cdot 7H_2O$ 0.1%, K_2HPO_4, 0.1%, sodium glutamate 0.1%, $FeSO_4 \cdot 7H_2O$ 0.01%, $MnSO_4 \cdot H_2O$ 0.0001%, $ZnSO_4 \cdot 2H_2O$ 0.0001%, adjusted to pH 7.0 with 1 M NaOH solution. The broth was incubated at 28°C with an aeration of 1.75 litres per minute with an agitation of 500 rpm. At 24 hours the broth was fed with 60 mg of **I**, 13-*epi*-selamectin aglycone, dissolved in a minimum volume of methanol and the fermentation harvested at 144 hours.

The culture broth from the mini-jar was extracted with ethyl acetate (5 litres) and then the solvent layer concentrated to gum solid (4.2 g). The extract was then dissolved in methylene chloride and applied to a column of Kieselgel 60 (160 g, 230 mesh) which had previously been conditioned with hexane. The column was sequentially eluted with methylene chloride (300 ml), 1 : 4 ethyl acetate : methylene chloride (200 ml), 1 : 1 ethyl acetate : methylene chloride (200 ml) and ethyl acetate (500 ml). 15 ml fractions were collected and compared by TLC using a developing solvent of ethyl acetate on Merck Kieselgel 60 F254 plates with detection by UV. The fractions (39~46) containing the component of interest (Rf 0.65) were combined and evaporated to dryness to yield 161 mg of enriched solid.

The solid was further purified by preparative reverse phase HPLC using a Zorbax 7 μ ODS column (21.2 mm× 25 mm) with a gradient mobile phase of 30 : 70 water : methanol to 100% methanol over 70 minutes and then at 100% methanol to 80 minutes with a flow rate of 6 ml/minute. The UV detector was set at 243 nm and fractions were collected at minute intervals.

The component of interest was eluted at 61 minutes and the fractions 60~62 were combined and evaporated to dryness to yield 13.2 mg of a white solid (22% yield). Proton NMR spectra were recorded on a Varian UNITY plus 500 NMR spectrometer or a Bruker 300 NMR spectrometer. Mass spectral data was collected on a Trio 1000 thermospray mass spectrometer.

Results and Discussion

The *S. avermitilis* mutant, 15-63, contains no functional branched-chain 2-oxo acid dehydrogenase activity and is thus unable to grow with isoleucine, valine and leucine as sole carbon sources.[2] Moreover it is unable to biosynthesise avermectins unless branched short chain fatty acids are supplied during the course of the fermentation.[3] We have shown that this organism is capable of functionalising the 13-OH function of **I** with an oleandrose when fed with the 13-*epi*-selamectin aglycone **I**. The epimerisation is described elsewhere.[4] (Fig. 1)

Following the addition of **I** to this organism all the starting material disappeared and three bioconversion products appeared, unexpectedly the major of which was the 13-*epi*-α-oleandrosyl analogue of selamectin, **II** (Fig. 2). The presence of this monosaccharide is intriguing, as previous studies have shown that with naturally derived avermectin aglycones, which are the natural substrates for these glycosidation enzymes, the sole products when fed to this organism are disaccharides.[5] Significant changes in chemical shift of the surrounding protons suggested that **II**

Table 1. Proton NMR data for selamectin and **II**.

Atom	Selamectin in $CDCl_3$ δ_H (J in Hz, 500 MHz)	(II) in $CDCl_3$ δ_H (J in Hz, 300 MHz)
2	3.4 (1H, m)	3.37 (1H, m)
3	5.83 (1H, dq, 2.5,1.4)	5.73-5.9 (1H, m)
6	4.67 (1H, s)	4.65 (1H, s)
8a	4.76 (1H, dd, 2.6,14.2)	4.76 (1H, dd, 2.6,14.2)
	4.69 (1H, dd, 2.6,14.2)	4.69 (1H, dd, 2.6,14.2)
9	5.95 (1H, dt, 10.5, 2.6)	5.73-5.9 (1H, m)
10	5.74 (1H, dd, 10.5, 15.2)	5.73-5.9 (1H, m)
11	5.76 (1H, dd, 9.5, 15.2)	5.3-5.4 (1H, m)
12	2.52 (1H, ddq, 2.5, ~9, ~7)	2.4 (1H, m)
13	3.96 (1H, m)	3.48 (1H, m)
15	4.98 (1H, br. d, 11)	5.2 (1H, t, 8.7)
16	2.3 (1H, m)	2.15-2.3 (2H, m)
	2.26 (1H, m)	
17	3.65 (1H, ddt, 2.1, 4.7, 11)	3.35-3.55 (1H, m)
18	1.79(eq) (1H, m)	1.7(eq) (1H, m)
	0.82(ax) (1H, dd, 11.5, 12.4)	0.8(ax) (1H, m)
19	5.42 (1H, tt, 5, 11.5)	5.3-5.4 (1H, m)
20	1.98(eq) (1H, ddd, 1.6, 5, 11.8)	1.95(eq) (1H, m)
	1.37(ax) (1H, t, 11.8)	1.3(ax) (1H, m)
22	1.65 (1H, m)	
	1.47 (1H, m)	
23	1.49 (2H, m)	
24	1.52 (1H, m)	1.5(1H, m)
25	3.07 (1H, br. d, ~9)	3.0 (1H, br. d, ~9)
4-Me	1.94 (3H, dd, 1.4, 2.4)	1.92 (3H, br. s)
12-Me	1.15 (3H, d, 7)	1.06 (3H, d, 7)
14-Me	1.5 (3H, br. s)	1.55 (3H, br. s)
24-Me	0.79 (3H, br. d, 5.8)	0.8(3H, br. d)
26	1.53 (1H, m)	
27	1.31-1.14 (1H, m)	
	1.8 (1H, m)	
28	1.31-1.14 (1H, m)	
	~1.64 (1H, m)	
29	1.42 (1H, m)	
	~1.6 (1H, m)	
30	1.6 (1H, m)	
	1.23 (1H, m)	
31	1.31-1.14 (1H, m)	
	1.8 (1H, m)	
1'	4.83 (1H, br. d, 3.8)	4.9 (1H, br. d)
2'	2.24(eq) (1H, m)	2.25 (eq) (1H, m)
	1.54(ax) (1H, m)	1.45 (ax) (1H, m)
3'	3.58 (1H, ddd, 4.8, 8.9, 11.4)	3.45 (1H, m)
4'	3.17 (1H, dt, 1.8, 9.1)	3.13 (1H, t, 9.7)
5'	3.87 (1H, dq, 9.5, 6.2)	3.5 (1H, m)
3'-OMe	3.48 (3H, s)	3.4 (3H, s)
5'-Me	1.27 (3H, d, 6.2)	1.15 (3H, d, 7)
5-NOH	8.69 (1H, br. s)	8.6 (1H, br. s)
7-OH	3.96 (1H, s)	3.9 (1H, br. s)
4'-OH	2.77 (1H, br. d, 2)	2.68 (1H, br. s)

Fig. 1. C-13 inversion.

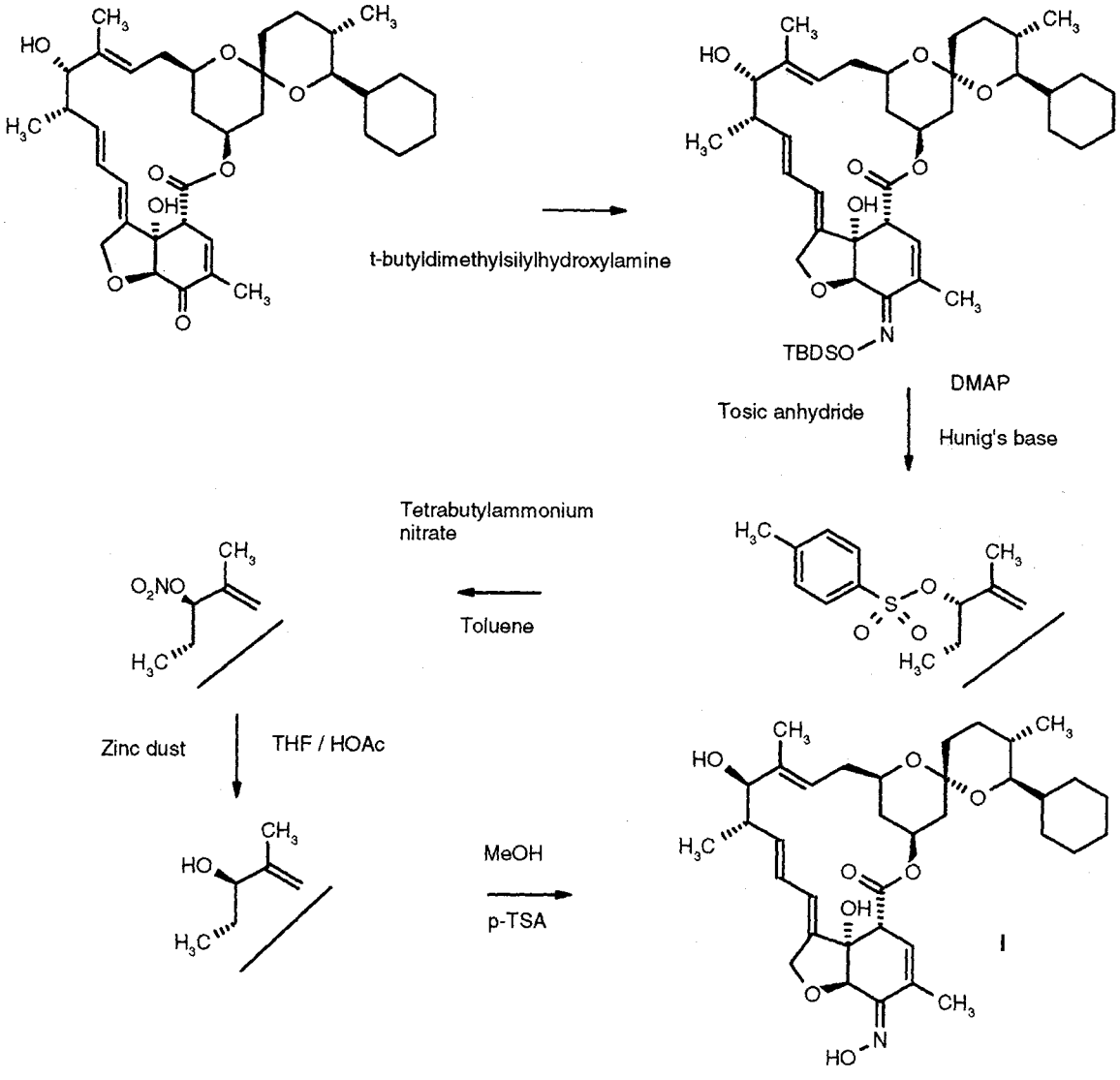


Fig. 2. Biotransformation.

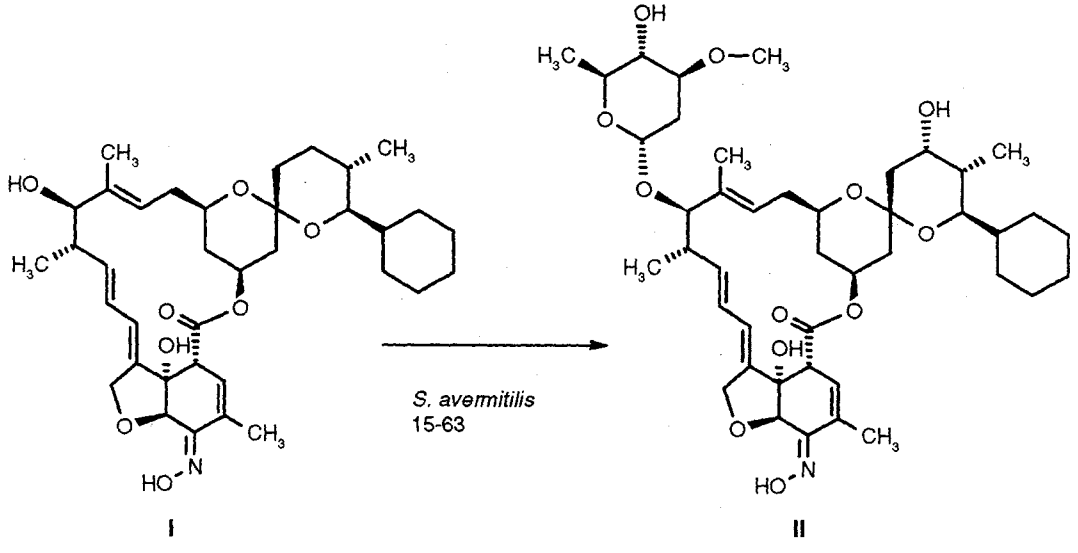
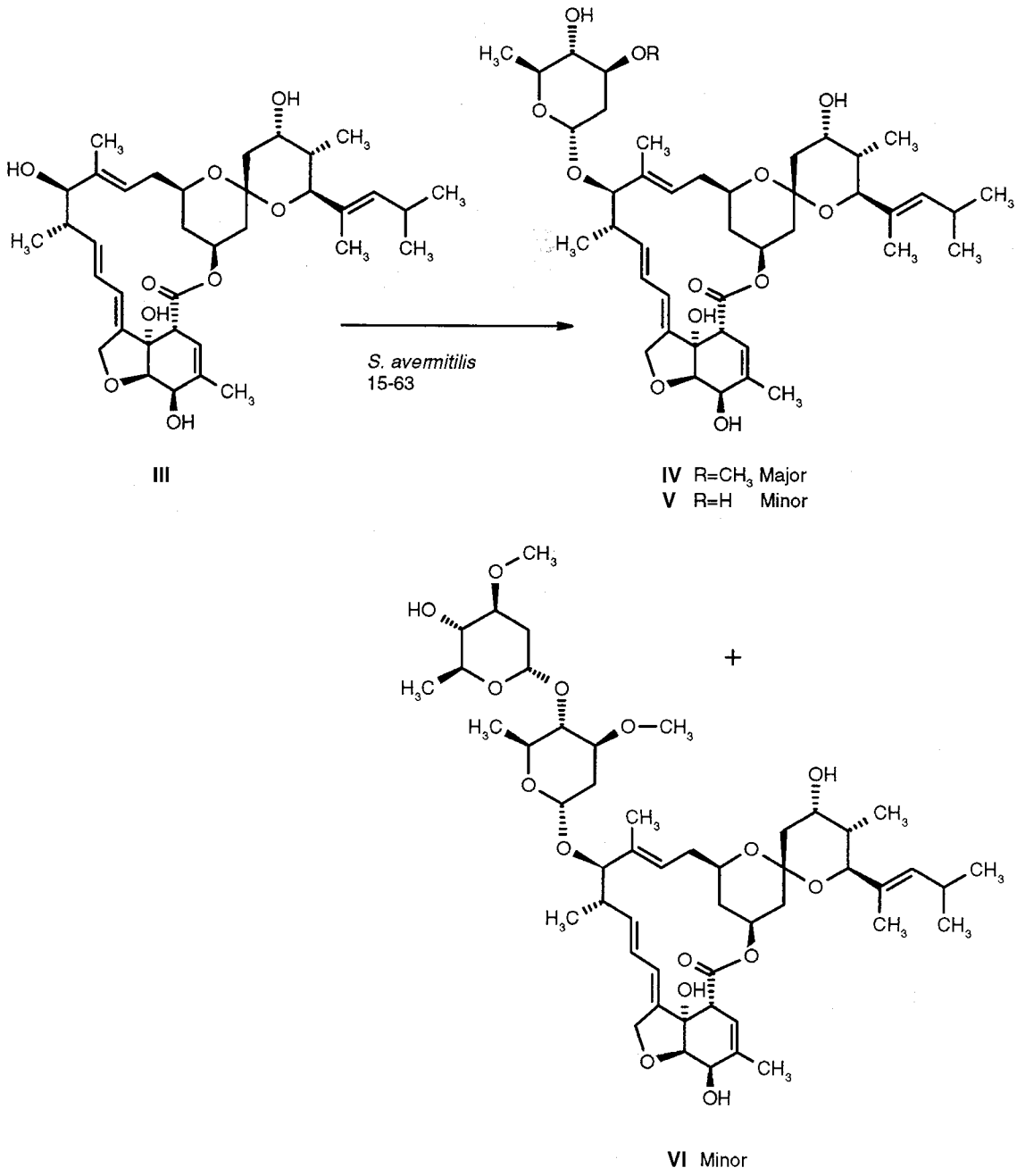
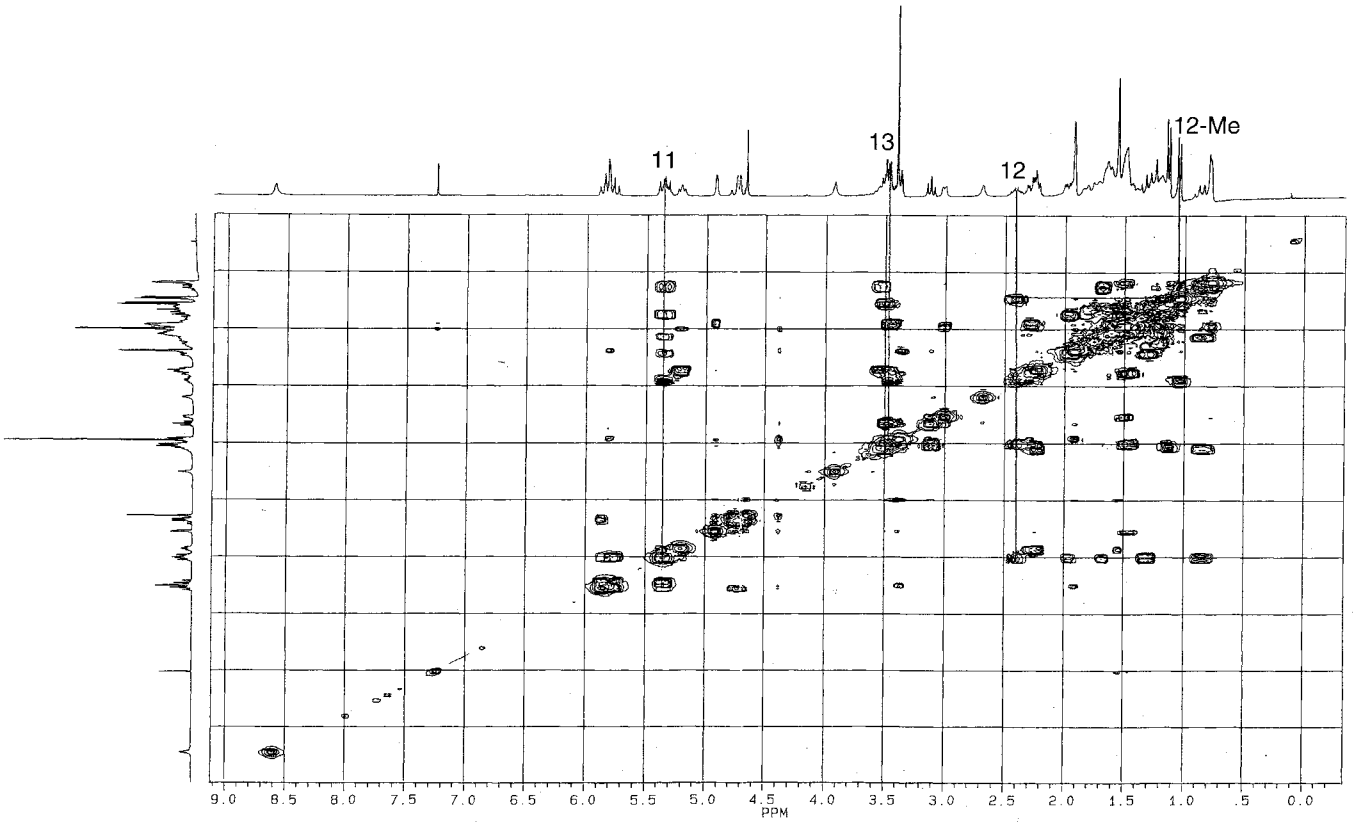


Fig. 3.



had a different stereochemistry at the 13 position from selamectin. Thus the 11-, 12-, 13- and 15-protons and the 5′- and 12-methyl protons all had chemical shifts altered by up to 0.46 ppm compared with those found in selamectin. (see Table 1). The assignments of these protons were confirmed by inspection of the 2-dimensional proton NMR (COSY) of **II** (Fig. 4). The 12-proton signal can clearly be seen to couple with the 11 and 13 protons and the 12-methyl group. The 13-proton signal was not sufficiently resolved at either 300 or 500 MHz to allow determination of the coupling constant with the 12-proton. However, in the NMR spectrum of **II** it had moved upfield by at least 0.46 ppm and could not be resolved from a number of protons which were attached to carbons adjacent to oxygen. TSP-MS data gave the expected molecular ion *m/z* 771 ([M+H$^+$]) for **II** which is the same as that for selamectin. HPLC assay[3] gave a retention time of 22.4 for **II** and 23.0 minutes for selamectin.

Fig. 4. 2D Proton NMR of **II**.



(13*R*)-Hydroxy-milbemycin **III**, prepared either by hydrolysis of the corresponding fermentation-derived (13*R*)-acyloxy milbemycin[6] or by oxidation of C-13 unsubstituted milbemycin LLF-28249α[7] were also fed to *S. avermitilis* 15-63 using the same protocol described above. Again the major biotransformation product was the corresponding (13*R*)-α-oleandrosyl-milbemycin **IV** and the minor products were identified as the 3′-*O*-demethyl-(13*R*)-α-oleandrosyl milbemycin **V** and the disaccharide, (13*R*)-α-oleandrosyl-α-oleandrosyl milbemycin **VI**. (Fig. 3) **VI** was also formed by re-feeding the monosaccharide **IV** to the fermentation, indicating that **IV** is an intermediate in the formation of **VI**.

All products showed at least 10× reduced activity in a flea *in vitro* assay compared with selamectin.

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