

ANTIMICROBIAL ACTIVITIES OF INDOLOCARBAZOLE AND BIS-INDOLE PROTEIN KINASE C INHIBITORS

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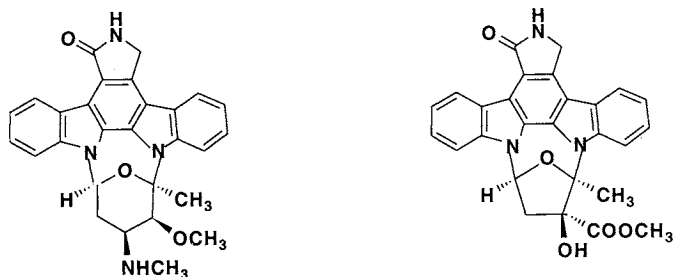
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The antimicrobial activities of twenty-two substances structurally related to staurosporine, aglycone in the indolocarbazole and bis-indole series were examined against *Streptomyces chartreusis* and *Streptomyces griseus*, *Bacillus cereus*, *Escherichia coli*, *Candida albicans* and *Botrytis cinerea*. Inhibition of sporulation was examined also on the two species of *Streptomyces*. Unlike literature reports for efficient protein kinase inhibitors, staurosporine and K-252a, no evident correlation could be found either between protein kinase inhibitory potencies and inhibition of sporulation of the *Streptomyces* species, or protein kinase inhibitory potencies and growth of all microorganisms tested. A weak activity against *C. albicans* was observed for the chloro-indolocarbazole compounds as already reported for structurally related substances from the cyanobacterium *Tolypothrix tjipanasensis*.

Streptomycetes are Gram-positive bacteria which form a spore-producing aerial mycelium. It was found recently that the microbial indolocarbazole metabolites, staurosporine¹⁾ and K-252a²⁾ (Fig. 1), inhibit aerial mycelium formation and antibiotic production by *Streptomyces griseus*³⁾. These compounds are known also as inhibitors of eukaryotic protein kinase C.

To determine if there was a general link between activity on protein kinase C and inhibition of spore formation, the *in vitro* growth inhibitory effect of twenty-two compounds in the indolocarbazole and bis-indole series were tested against *S. griseus* and *Streptomyces chartreusis*. The activities of these twenty-two compounds towards protein kinase C and protein kinase A have been determined previously; some of them were active, others not⁴⁻⁷⁾. Production of the secondary metabolite calcimycin (A 23187) by *S. chartreusis* was examined when the culture was incubated with an efficient protein kinase C inhibitor and with a compound inactive towards this enzyme. The antimicrobial activities against *Bacillus cereus*, *Escherichia coli*, *Candida albicans* and *Botrytis cinerea* were determined also.

Fig. 1.



Staurosporine IC_{50} PKC = 3 nM

K-252a IC_{50} PKC = 32.9 nM

Results and Discussion

Rebeccamycin **1** (laboratory stock sample) was isolated from cultures of *Saccharotrix aerocolonigenes*⁸. Compounds **2**~**22** were prepared by either total synthesis or hemisynthesis from rebeccamycin.

Compounds **2**~**11**, structurally related to staurosporine or K-252a aglycones, have different framework flexibilities and different functions (imide, amide and amide-alcohol) on the non-indolic heterocycle (Fig. 2)⁴.

Compounds **12**~**16** (Fig. 3) are amides in the indolocarbazole and bis-indole series in which the amide group is acyclic; these compounds, structurally related to those shown in Fig. 2, have no inhibitory effect on protein kinase C and protein kinase A, as the rigid cyclic structure of the amide group is compulsory for activity against these enzymes⁵.

Compounds **17**~**21** (Fig. 4) were obtained by hemisynthesis from rebeccamycin⁶.

Fig. 2.

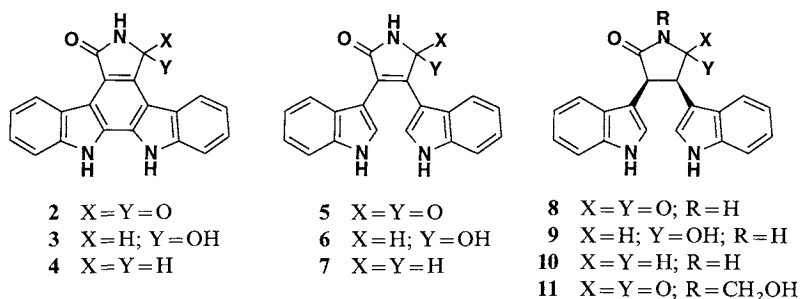


Fig. 3.

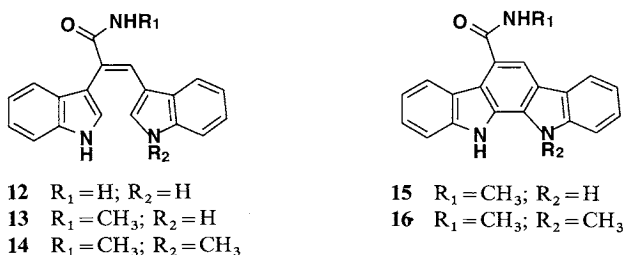
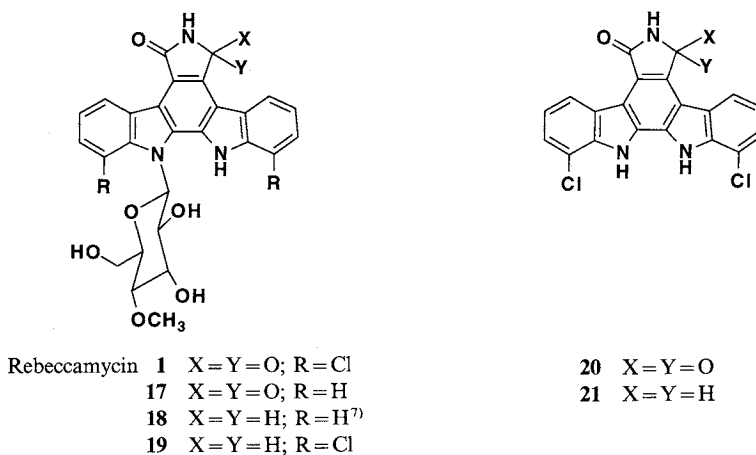


Fig. 4.



Compound **22** (Fig. 5), a biomimetic analog of K252a was prepared by coupling **2** with methyl-2,5-dimethoxy-tetrahydrofuran-3-carboxylate using *p*-toluenesulfonic acid (*p*-TsOH) in refluxing CH₂Cl₂.

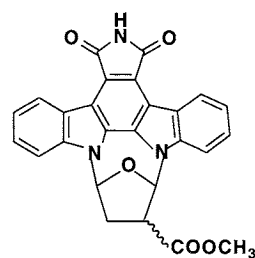
Methyl-2,5-dimethoxy-tetrahydrofuran-3-carboxylate was prepared from commercial 2,5-dimethoxy-tetrahydrofuran-3-carbaldehyde by oxidation with potassium permanganate in a *tert*-butylalcohol - aqueous NaH₂PO₄ system according to the method described by ABIKO *et al.*⁹⁾, on carbaldehyde-tetrahydropyran moieties.

Since both staurosporine and K-252a allow growth of *S. griseus* even at high concentrations but inhibit its aerial mycelium formation, we examined the inhibition of both growth and sporulation of the two species of *Streptomyces* tested and the inhibition of growth of the other microorganisms tested. The results are summarised in Table 1.

No correlation was observed between activity against protein kinase C (PKC) and antimicrobial activity on *B. cereus*. Of the few compounds inactive towards *B. cereus* **2**, **15**, **17**, **20** and **22**, **2** and **22** are efficient PKC inhibitors while **15**, **17** and **20** are not.

Few of the products tested exhibited antifungal

Fig. 5.

Table 1. Inhibitory potencies of compounds **1**~**22** against protein kinase C (PKC) and protein kinase A (PKA).

Product	IC ₅₀ PKC (μM)	IC ₅₀ PKA (μM)	<i>S. chartreusis</i> NRRL 11407		<i>S. griseus</i> ATCC 23345		<i>E. coli</i> ATCC 11303	<i>B. cereus</i> ATCC 14879	<i>C. albicans</i> IP 444	<i>B. cinerea</i> DSM 877
			g.	sp.	g.	sp.				
			1 ^a	>100	>100	+				
2	44.7	60	-	-	-	-	-	-	-	-
3	22.1	34	++	++	++	+	-	++	±	±
4	2.45	25.7	-	-	-	-	-	+	-	-
5	0.087	2.7	+++	+++	+++	+++	-	++	-	-
6	7.6	>50	±	-	+	-	-	+	-	-
7	2.5	32	++	+	+	+	-	+	-	-
8	>100	>100	++	+++	++	+++	-	+	-	±
9	>100	>100	+	-	±	-	-	+	-	-
10	>50	>50	+	++	+	-	-	+	-	-
11	16	>100	+++	+++	++	++	-	+	-	-
12	>100	>100	++	+++	++	++	-	++	-	-
13	>100	>150	+	±	+	±	-	+	-	-
14	>100	>100	+	++	+	+	-	±	-	-
15	>100	>100	++	-	+	-	-	-	-	-
16	>100	>100	+	-	+	-	-	+	-	-
17	>100	>150	±	-	-	-	-	-	-	-
18	3.7	n.d.	+	-	-	-	-	++	-	-
19	28.8	>150	++	±	++	+	-	+	-	-
20	>50	>100	-	-	-	-	-	-	+	-
21	>100	>100	++	++	++	+	-	+++	+	±
22	4.85	34.7	-	-	-	-	-	-	-	-

(IC₅₀ μM); *in vitro* growth inhibitory effect against different microorganisms (for the 2 species of *Streptomyces*: g. means inhibition of growth, sp. means inhibition of sporulation). (+++), (++) , (+), (±) and (-) mean strong, medium, weak, very weak and no activity. The size of zones of growth inhibition was >12 mm (+++), 10~12 mm (++) , 7~9 mm (+), 6~7 mm (±).

^a Antimicrobial activities are in agreement with literature.⁹⁾

Fig. 6. Effects of compounds **5** and **12** on growth and sporulation of *S. chartreusis*.

a) Photograph of Petri plates; b), c), d) scanning electron microscopy photographs. b) *S. chartreusis* as control, bar represents 5.0 μm . c) Inhibition of sporulation by **5**, bar represents 5.0 μm . d) Inhibition of sporulation by **12**, bar represents 5.0 μm .

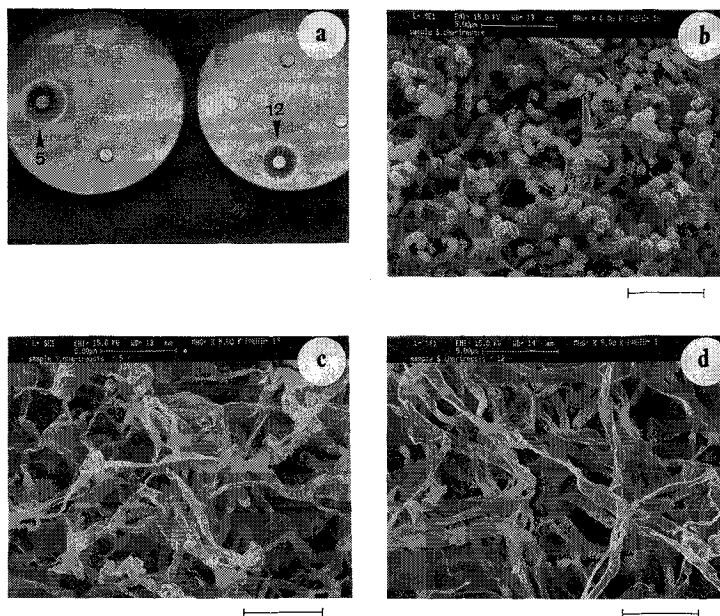
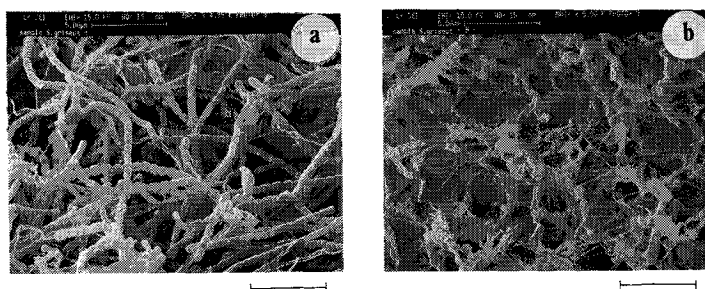


Fig. 7. Effects of compound **5** on sporulation of *S. griseus*.

Scanning electron microscopy photographs. a) *S. griseus* as control, bar represents 5.0 μm . b) Inhibition of sporulation by **5**, bar represents 5.0 μm .



activities; **20** and **21**, which were active against *C. albicans* are chloro-indolocarbazole systems, and their fungicidal activity was comparable to that observed with chloro-indolocarbazole tjipanazoles isolated from *Tolypothrix tjipanansensis*¹⁰. Examples of the effects of compounds **5** (active against PKC) and **12** (inactive against PKC) on growth and sporulation of *S. chartreusis* are shown in Fig. 6. Fig. 7 shows the activity of **5** on *S. griseus*.

In vitro phosphorylation reactions revealed multiple phosphorylated proteins in *S. griseus*³. Staurosporine and K-252a which are eukaryotic protein kinase inhibitors (staurosporine: IC_{50} PKC 3 nM and ranging from 3 to 30 nM on other protein kinases¹¹, K-252a IC_{50} PKC 32.9 nM²) also inhibit the phosphorylation of several cellular proteins in *S. griseus*. These microbial metabolites inhibit aerial mycelium

formation and pigment production by *S. griseus*. This suggests a relationship between protein phosphorylation and both aerial mycelium formation and pigment formation. Among the compounds implicated in aerial mycelium formation of different *Streptomyces* are A-factor and the small protein SapB. A-factor or related compounds are secreted by many species of *Streptomyces* and thought to diffuse freely into cells, bind a cytoplasmic protein and inactivate the protein's ability to repress sporulation¹²⁾. SapB protein production, triggered by extracellular signals, plays an important role in the formation of aerial hyphae¹³⁾. It appears that staurosporine and K-252a inhibit the formation of the compounds involved in sporulation or interact with them leading to their inactivation, and so it was concluded that these mechanisms are implicated in the inhibition of protein phosphorylation induced by the protein kinase C inhibitors, staurosporin or K-252a. In this light, the results we obtained on the two strains of *Streptomyces* were quite unexpected:

1. Unlike staurosporine and K-252a, the active PKC inhibitors **3**, **5**, **7**, **11**, **18**, **19**, which inhibit the sporulation, also inhibited the growth of the *Streptomyces* species tested.
2. Efficient PKC inhibitors **2**, **4**, and **22** had no activity on the *Streptomyces* species tested.
3. **8** and **12** are inactive against PKC, but very strongly inhibited growth close to the disk and sporulation at slightly lower concentrations.
4. **1**, **9** and **16**, inactive against PKC, inhibited only growth.
5. Only compound **20** was both inactive on PKC and on the *Streptomyces* species.

Since staurosporine, K-252a and the active compounds tested interact with the ATP binding site of the catalytic domain common to all protein kinases, they exhibit no marked specificity towards these enzymes, and so protein kinase C, assuming it is implicated in the inhibition of sporulation, it's probably not the sole enzyme system involved.

Importantly, staurosporine and K-252a are more active protein kinase inhibitors (IC₅₀ in the nM range) than any of our compounds, and yet do not inhibit growth at the same concentration, in contrast to **5**, the strongest protein kinase C inhibitor tested here. Since **8** and **12**, which are inactive against PKC, inhibit the sporulation of *S. chartreusis* and *S. griseus*, the mechanism involved in the inhibition of sporulation does not seem directly correlated with the inhibition of protein phosphorylation by PKC.

The results obtained with **2**, **4** and **22**, which are active compounds against PKC but inactive on the microorganisms tested, are surprising. The lack of activity on *Streptomyces* could be due to absence of penetration into the cells or absence of diffusion through the gelose or metabolism by the cells. To investigate this, cultures of *S. chartreusis* were incubated with compounds **4** and **5** which are both active against PKC; **5** inhibited sporulation while **4** did not. After four days of incubation, the cultures were extracted as indicated in the experimental section; **5** was recovered unchanged while metabolism of **4** was observed from TLC and ¹H NMR spectrum. Metabolism of **4** by the microorganism may therefore explain its inactivity.

As described in the literature³⁾, inhibition of sporulation seems to parallel that of secondary metabolite formation. Accordingly, we examined the production of the ionophorous antibiotic A23187 by *S. chartreusis* after incubation of cultures with compounds **4** and **5** comparatively to a blank. In the blank, the production of A23187 began after two days, peaking after four days. When cultures were incubated with **5**, A23187 production was arrested until the fourth day and then began slowly. Incubation with **4** led to both reduced growth and A23187 production, and biotransformation of the PKC inhibitor.

It would be relevant to examine if the amides **12**, **13**, **14**, which are inactive against PKC but inhibit

the sporulation of *S. chartreusis*, are metabolised into efficient PKC inhibitors. This would explain their effect on sporulation. If not, it may be that the inhibition of sporulation does not necessarily imply the inhibition of protein phosphorylation by PKC. We are currently investigating this possibility. These results also show that the inhibition of sporulation of *Streptomyces* species cannot be used as a rapid and cheap early test for the inhibition of PKC.

Experimental

IR spectra were recorded on a Perkin-Elmer 881 spectrometer (ν in cm^{-1}), NMR spectra on a Bruker MSL 300 (^1H : 300 MHz, ^{13}C : 75.45 MHz) (chemical shifts δ in ppm, the following abbreviations are used: singlet (s), doublet (d), triplet (t), quadruplet (q), multiplet (m), tertiary carbons (C tert), quaternary carbons (C quat)). Mass spectra (EI) were determined at the Service Central d'Analyses, CNRS (Vernaison) on a VG.ZAB SEQ and at CESAMO (Talence) on a high resolution FISOONS Autospec-Q spectrometer. Chromatographic purifications were performed with flash Geduran SI 60 (Merck) 0.040~0.063 mm.

2,5-Dimethoxy-tetrahydrofuran-3-carboxylic Acid

Commercial 2,5-dimethoxy-tetrahydrofuran-3-carbaldehyde (1 g; 6.25 mmol) in *tert*-BuOH (37 ml) was diluted in a 5% NaH_2PO_4 buffer (pH 4.4; 25 ml). A 1 M KMnO_4 solution (37.5 ml; 37.5 mmol) was added. The mixture was stirred for 10 minutes at room temperature, neutralised with saturated aqueous Na_2SO_3 then acidified to pH 3.5 with diluted HCl. After extraction with AcOEt, purification by flash chromatography (AcOEt-cyclohexane 50:50) afforded the acid as colourless crystals (542 mg; 3.08 mmol; 49% yield). ^1H NMR (60 MHz, CDCl_3): 2.3 (2H, m, $\text{C}_4\text{-H}_2$); 3.1 (1H, m, $\text{C}_3\text{-H}$); 3.4 (6H, s, OCH_3); 5.2 (2H, m, $\text{C}_2\text{-H}$, $\text{C}_5\text{-H}$); 10.2 (1H, br s, COOH).

Methyl-2,5-dimethoxy-tetrahydrofuran-3-carboxylate

To the above acid (2.66 g; 15.1 mmol) in methanol (10 ml) was added CH_2N_2 in ether until a yellow colour, indicating an excess of diazomethane persisted. After evaporation of the solvents, purification by flash chromatography (AcOEt-cyclohexane 50:50) afforded the ester as a colorless oil (2.810 g; 14.8 mmol; 98% yield). IR (KBr) $\nu_{\text{C=O}}$ 1740; Mass (EI) ($\text{M}-\text{OCH}_3$)⁺ 159 (30%). ^1H NMR (60 MHz, CDCl_3): 2.3 (2H, m, $\text{C}_4\text{-H}_2$); 3.1 (1H, m, $\text{C}_3\text{-H}$); 3.4 (6H, s, OCH_3); 3.9 (3H, s, COOCH_3); 5.3 (2H, m, $\text{C}_2\text{-H}$, $\text{C}_5\text{-H}$). ^{13}C NMR (15 MHz, CDCl_3): 32.3; 33.7; 35.1 (C_4); 47.3; 48.8; 49.7; 51.9; 52.3; 55.0; 55.3; 55.5 (COOCH_3 , OCH_3 , C_3); 104.1; 105.0; 105.2; 106.2; 107.7 (C_2 , C_5); 169.5; 170.0; 171.9; 172.7 (COOCH_3).

10-Methyloxycarbonyl-9,10,11,12-tetrahydro-9,12-epoxy-(1*H*)-diindolo[1,2,3,f-g:3',2',1'-k-j]-pyrrolo-[3,4-*i*][1,6]-benzodiazocin-1,3-(2*H*)-dione (22)

To compound **2** (258 mg; 0.794 mmol) in CH_2Cl_2 (90 ml) were added the above ester (323 mg; 1.7 mmol; 2.1 equiv) and catalytic amounts of *p*-TsOH. The mixture was refluxed for 20 hours then poured into water and extracted with AcOEt. Purification by flash chromatography (AcOEt-cyclohexane 20:80) afforded **22** as a yellow fluorescent solid (23 mg; 0.051 mmol; 6% yield). IR (KBr) $\nu_{\text{N-H}}$ 3200 $\nu_{\text{C=O}}$ 1750; mp 253~257°C. Exact mass calculated for $\text{C}_{26}\text{H}_{17}\text{N}_3\text{O}_5$: 451.1168 found 451.1146. ^1H NMR (300 MHz, $\text{DMSO}-d_6$): 2.0~2.2 (2H, m, $\text{C}_{11}\text{-H}_2$); 2.9~3.2 (1H, m, $\text{C}_{10}\text{-H}$); 3.40 and 3.45 (3H, 2s, COOCH_3); 6.30 (1H, t, $J=8$ Hz); 7.12 (1H, s); 7.38 (2H, m); 7.54 (2H, t, $J=9$ Hz); 7.71 (1H, d, $J=9$ Hz); 7.94 (1H, d, $J=9$ Hz); 9.11 (1H, d, $J=9$ Hz); 9.16 (1H, d, $J=9$ Hz); 11.02 and 11.10 (1H, 2s, NH). ^{13}C NMR (75.47 MHz, $\text{DMSO}-d_6$): 30.1 (C_{11}); 50.9 (COOCH_3); 56.0; 61.3; 65.4 (C_9 , C_{10} , C_{12}); 109.9; 112.4; 120.3; 120.8; 124.4; 124.8; 126.9 (2C) (C tert); 116.0; 117.5; 119.2; 121.2; 128.0; 128.9; 140.2; 141.3 (C quat); 167.5; 170.3; 170.9 (C=O).

Antimicrobial Tests

Six strains were tested: 3 Gram-positive bacteria (*B. cereus* ATCC 14879, *S. chartreusis* NRRL 11407, *S. griseus* ATCC 23345), a Gram-negative bacterium (*E. coli* ATCC 11303) a yeast (*C. albicans* 444 from Pasteur Institute) and a filamentous fungus (*B. cinerea* DSM 877). Antibacterial activity was determined

by the conventional paper disk (Durieux, No. 268, 6 mm in diameter) diffusion method using the following nutrient media: Mueller-Hinton (Difco) for *B. cereus* and *E. coli*, Sabouraud agar (Difco) for *C. albicans* and *B. cinerea* and Emerson agar (0.4% beef extract, 0.1% yeast extract, 0.4% peptone, 1% dextrose, 0.25% NaCl, 2% agar, pH 7.0 (Difco)) for the *Streptomyces*. Growth inhibition was examined after 24 hours incubation at 27°C (37°C for *E. coli*). Inhibition of sporulation was examined 3 to 5 days later. Products **1**~**22** were dissolved in DMSO and a paper disk containing each of the products (300 µg) was placed on regeneration medium seeded with spores.

For the control of calcimycin (A 23187) production by *S. chartreusis* in the presence of protein kinase C inhibitor, the inhibitor (600 µg in 10 ml of DMSO) was introduced into the culture medium (100 ml)¹⁴. After 1, 2, 3, 4 days of incubation, the medium was filtered off, the mycelium was extracted with ethanol and the filtrate with ethyl acetate, after concentration, the A23187 production was examined by TLC and compared with a blank.

Scanning Electron Microscopy

Pieces of mycelium were cut out and fixed under osmium tetroxide vapour for 15 hours, then allowed to dry for 12 hours. After sputter coating with gold for 2 minutes under vacuum, the mycelium was examined under a Cambridge stereoscan 360 scanning electron microscope.

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