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SCREENING OF CYTOTOXIC ACTIVITIES OF *STRYCHNOS* ALKALOIDS (METHODS AND RESULTS)

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Summary

The potential cytotoxic activities of 46 alkaloids isolated from different *Strychnos* species were tested on different cancer or normal cells cultured in vitro. The authors used a relatively simple microtest which gives good reproducibility. Most of the active compounds belong to the usambarane skeleton but other structure-activity relationships are being discussed.

Introduction

Extensive studies in our laboratory on the constituents of various African *Strychnos* species (*S. variabilis* De Wild., *S. usambarensis* Gilg, *S. gossweileri* Exell, *S. icaja* Baill.) have led to the isolation and structure determination of numerous alkaloids (Bisset, 1968; Angenot, 1974, 1975, 1976; Kambu et al., 1979; Coune, 1980; Angenot and Tits, 1981; Coune et al., 1984; Caprasse, 1983). They have been performed especially on medicinal and toxic plants collected in Central Africa (Rwanda and Zaire). Some of them e.g. *S. usambarensis* Gilg and *S. icaja* Baill., were ingredients of arrow poisons.

In a recent study correlating results obtained from the N.C.I. plant anti-tumor screening and selected types of folkloric uses, it would appear that one could increase by a factor of about five, the rate of plant species showing cytotoxicity or antitumor activity when selecting plants used as arrow poisons (Farnsworth and Kaas, 1981).

Recently some of the purified alkaloids were tested for in vitro and in vivo cytotoxic activities against B16 melanoma, Ehrlich ascites tumor cells and hepatoma cells. An antimitotic activity was demonstrated at 1—10 or 50 μg/ml (Bassleer et al., 1982; Tits et al., 1984).

We decided to undertake a screening of potential cytotoxic activities of 46 alkaloids available in our laboratory (indole or indoline, tertiary or

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quaternary, monomeric or dimeric) with the aim of suggesting structure-activity relationships, selecting the most active compounds and looking for substances of lower general toxicity.

Material and methods

Chemistry

The chemical structures of the tested alkaloids are shown in Figs. 1—7.

The quaternary alkaloids were in the chloride form, except serpentine hydrogenotartrate, and methylharmalane iodide.

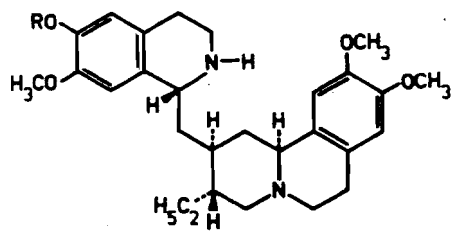
Tertiary alkaloids were converted to their acetates to increase their solubility in water.

For all the alkaloids tested, a stock solution was prepared by dissolving a quantity of salt corresponding to 2 mg of alkaloid base in 20 ml distilled water and filtration through a 45 μm micropore membrane. The solutions were added to the liquid culture medium to give a final concentration of 1—10 or 25 μg/ml.

Methods

Cytotoxic activities were tested in NUNC 96-well plates. In each well 0.1 ml medium containing a definite number of cells (depending on the type of cells used) was inoculated. Twenty four hours later, the medium was removed and replaced by 0.1 ml/well fresh medium containing 1, 10 or 25 μg/ml alkaloid.

Eight wells were used for each condition in order to avoid possible artefacts. After 72 h, the medium was eliminated and cell staining was performed on 2 wells for each condition with a 0.05% trypan blue solution in PBS; only dead cells were then stained and easily detected. The plates were then immediately observed under an inverted phase contrast microscope. This method gives a good idea of the average number of dead cells on the plate, but it has to be rapidly performed. In the other wells, cells were fixed by



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1a R = CH_3

1b R = H

Fig. 1.

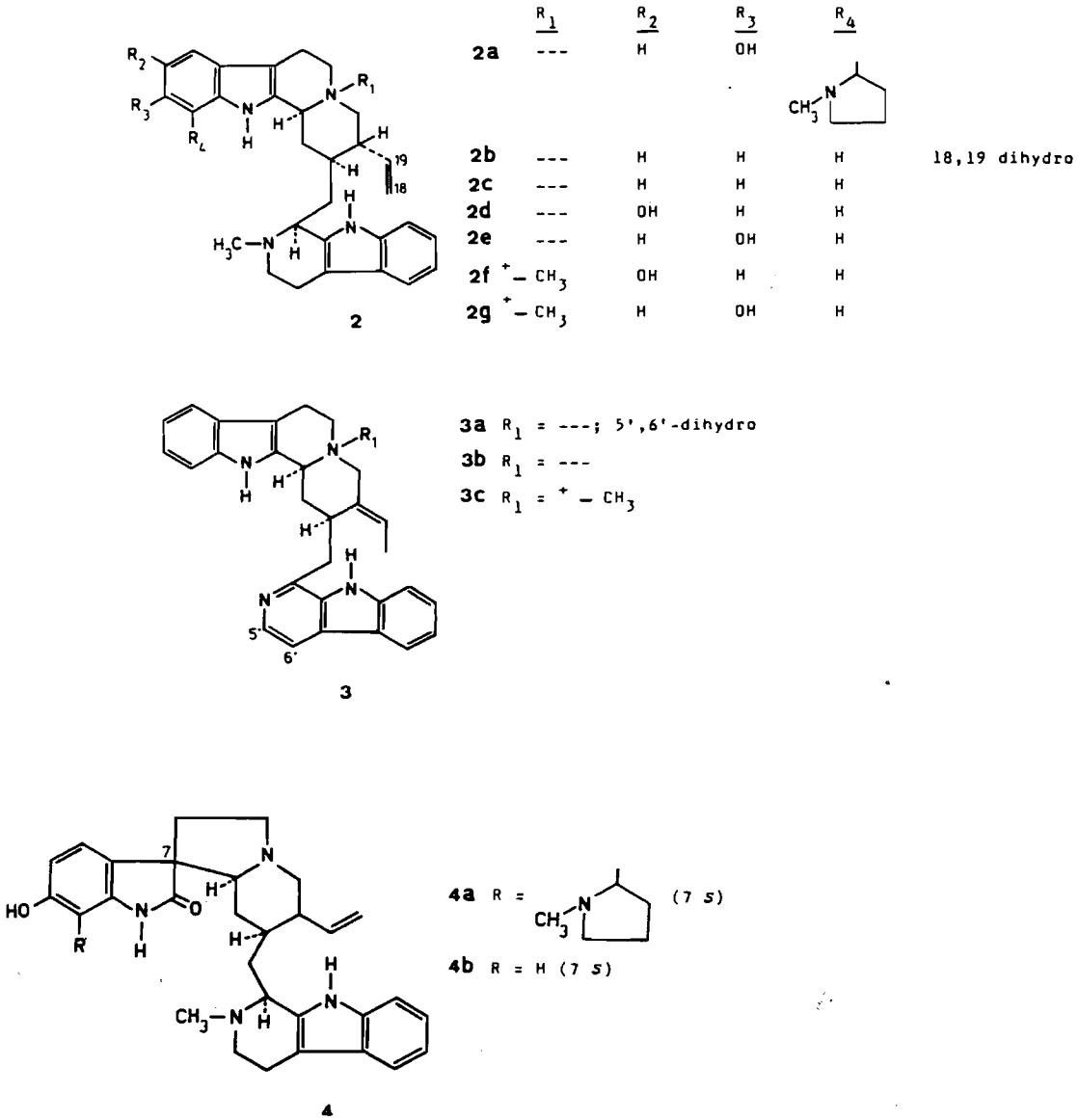


Fig. 2.

94% ethanol during 48 h and stained with a 0.2% toluidine blue solution in water.

Types of cells used

(1) Cultured B16 melanoma cells derived from C57BL mouse melanoma. Two series of experiments were performed:
(a) inoculation of 1000 cells per well and treatment 8 days later;
(b) inoculation of 5000 cells and treatment 24 h later.
In each case, control wells were prepared.
Nutrient medium: Gibco MEM 90% supplemented with 10% foetal calf serum and 100 units/ml penicillin.

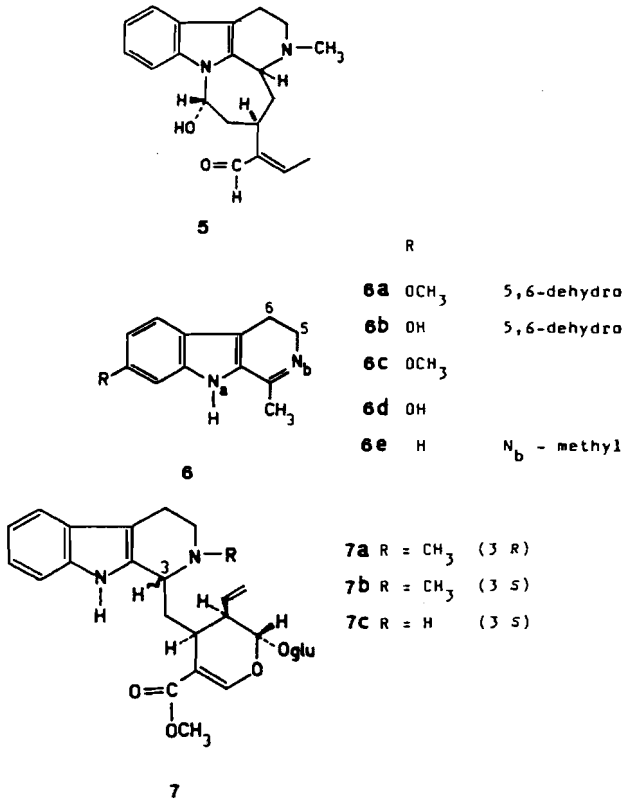


Fig. 3.

(2) Cultured Flow 2002 cells derived from normal embryonic human lungs; 15,000 cells per well were inoculated and treated 24 h later.
Culture medium: Gibco BEM 90% supplemented with 10% foetal calf serum and 100 units/ml penicillin.

(3) Cultured L 1210 cells derived from DB A/2 mouse ascites tumor. Inoculation of 80,000 cells per well on a polyornithine film for obtaining cell adherence in the well. The cells were treated 24 h after inoculation.
Culture medium: Flow RPMI 1640 medium 90% supplemented with 10% foetal calf serum and 100 units/ml penicillin.

(4) Cultured HeLa cells derived from a human carcinoma; inoculation of 5000 cells per well and treatment 24 h later.
Culture medium: Gibco MEM 90% complemented with 10% foetal calf serum and 100 units/ml penicillin.

Results and discussion

The results are presented in Table 1.

Determination of relative activity was performed comparing the number of cells and the percentage of dead and alive cells with controls. These determinations were carried out after 24 h by observing living cells and after 72 h after either trypan blue or toluidine blue staining.

We considered a molecule as active either when the number of cells strongly

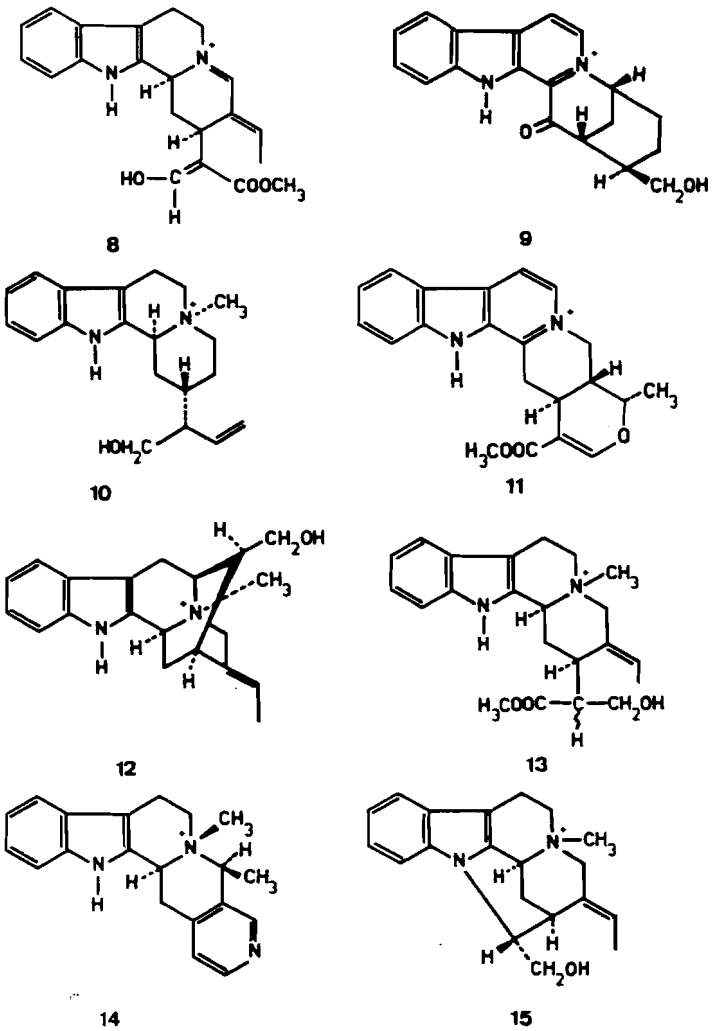


Fig. 4.

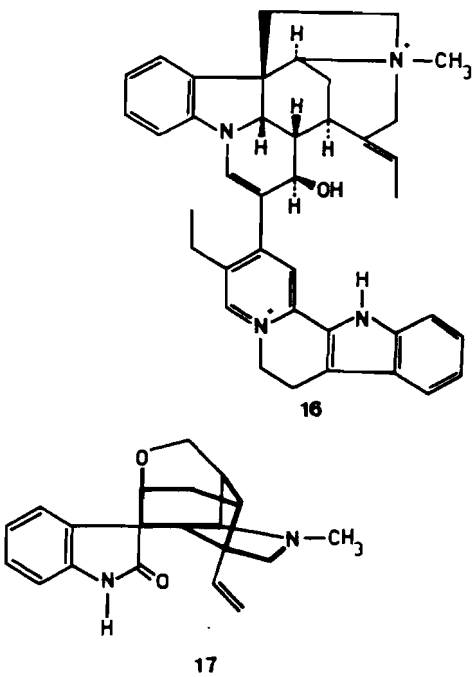
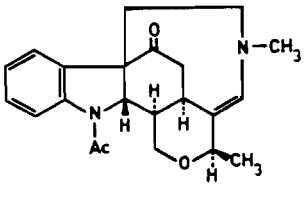


Fig. 5.



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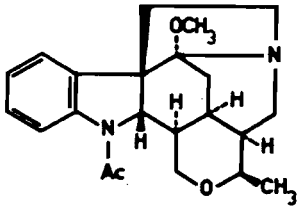

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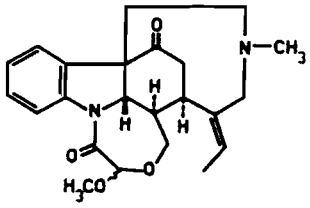

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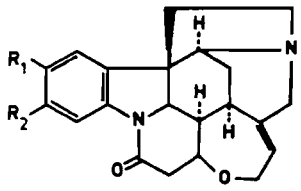

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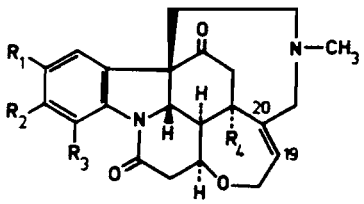
	R_1	R_2
21a	H	H
21b	H	OCH_3
21c	OCH_3	OCH_3



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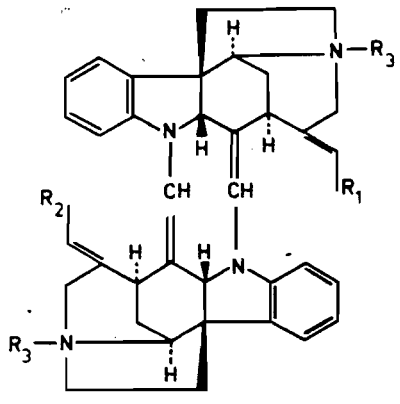
	R_1	R_2	R_3	R_4	
22a	H	H	OH	H	
22b	OCH_3	OCH_3	H	H	19,20-α-epoxy
22c	OCH_3	OCH_3	H	OH	19,20-α-epoxy
22d	H	H	H	H	

Fig. 6.



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23a $R_1 = R_2 = CH_3$ R_3 = ---

23b $R_1 = R_2 = CH_2OH$ $R_3 = {}^{+} - CH_2 - CH = CH_2$

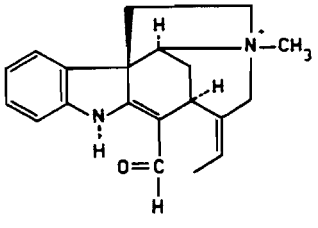

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Fig. 7.

decreased or when the average number of dead cells was higher than in the control cells.

From a general point of view, our results show that this test offers a good reproducibility.

Emetine and strychnopentamine were used as references so that correlations could be made with results coming from other work based on different methods.

Most of the active compounds had a usambarane skeleton (strychnopentamine (2a), dihydro)-usambarensine (3a3b), (dihydro)-usambarine (2b,2c), 10- or 11-hydroxy-usambarine (2d,2e), strychnophylline (4a)). This fact can be related to the relationship between the structure of these alkaloids and emetine (1a), all of which result from condensation of a monoterpenoid unit and an amino unit: tryptamine for the *Strychnos* alkaloids and tyramine for emetine. Emetine has well-known cytotoxic activity but it also has a high general toxicity (Suffness and Douros, 1980). It is the reason why it was thought possible to find less toxic compounds among these substances.

When comparing the activities of these usambarane derivatives some differences appear. Strychnopentamine (2a), the most active one, has a *N*-methyl-pyrrolidine group on C-12, while 11-hydroxy-usambarine (2e) has exactly the same structure but without this substitution. The results show a certain loss of activity for this last compound. The presence of a *N*-methyl-pyrrolidine substitution on C-12 also differentiates strychnophylline (4a) from strychnofoline (4b) with a greater loss of activity when lacking the *N*-methyl-pyrrolidine group.

We can also point out that the 5',6'-dihydro derivative (3a) is more active than the one with the extra double bond.

Another thing to be pointed out is that quaternarisation of the alkaloids strongly decreases the cytotoxic activity, e.g. there is a loss of activity when comparing usambarensine (3b) with N_b-methyl-usambarensine (3c). That is not surprising, because such a fact was already shown with a quaternary derivative of ellipticine which has a lower activity in vitro but a higher activity in vivo (Le Pecq and Paoletti, 1982).

Other skeletons were also analysed related to serpentine (11) for example, for which a patent has been taken out (Beljanski and Bugiel, 1978) because it seems to have a selective activity against cancer cells but these alkaloids do not have strong activity.

Another class of alkaloids tested here is β-carboline derivatives, the structure of which is close to that of ellipticine or olivacine which are well-known cytotoxic agents (Li and Cowie, 1974; Husson et al., 1981).

We have also tested other skeletons, e.g. strychnine derivatives, but none of them seems to be very active, except akagerine (5) which has a very particular structure and should be considered separately.

Conclusion

We have used a relatively simple test in vitro with which it is possible to

TABLE 1

RESULTS

0, no effect at all; +, decrease in the number of cells compared to controls, with the highest concentration after 72 h; ++, no effect at 1 μg/ml but many cells are dead, with the highest concentration after 72 h; +++, no effect at 1 μg/ml, but all cells are dead at 10 μg/ml after 24 h; ++++, good effect at 1 μg/ml after 72 h and all cells are dead at 10 μg/ml after 24 h; +++++, all cells are dead at 1 and 10 μg/ml after 24 h.

Name	Concentration (μg/ml)	B16	2002	L1210	HeLa
Emetine Fluka (1a)	1—10	+++++	+++++	+++++	+++++
Cephaeline Sandoz (1b)	1—10	+++++	++++	+++++	+++++
1. Indole alkaloids					
1.1 Usambarane skeleton					
1.1.1 Tertiary dimers					
Strychnopentamine (2a)	1—10	+++	++++	+++++	+++
Dihydro-usambarensine (3a)	1—10	+++	+++	+++++	+++
Dihydro-usambarine (2b)	1—10	+++	+++	+++++	+++
Usambarine (2c)	1—10	+++	+++	++++	+++
Usambarensine (3b)	1—10	+++	+++	+++	+++
10-Hydroxy-usambarine (2d)	1—10	+++	+++	+++	+++
11-Hydroxy-usambarine (2e)	1—10	+++	+++	+++	++
Strychnophylline (4a)	1—10	+++	+++	+++	+
Strychnofoline (4b)	1—10	0	0	+	0
1. 1.2. Tertiary and quaternary dimers					
N_b-Methyl-11-hydroxy-usambarine (2g)	1—10—25	++	0	+	0
N_b-Methyl-10-hydroxy-usambarine (2f)	1—10—25	+	0	+	0
N_b-Methyl-usambarensine (3c)	1—10—25	+	0	+	0
1.2. Tertiary monomers					
Akagerine (5)	1—10	++	+++	+++	++
Harmine Fluka (6a)	1—10	0	+	++	++
Harmol Fluka (6b)	1—10	0	0	++	++
Harmaline Fluka (6c)	1—10	0	0	+	0

Harmalol Fluka (6d)	1—10	0	0	0	0
Glycoalkaloids					
Isodolichantoside (7a)	1—10	0	0	++	0
Dolichantoside (7b)	1—10	++	0	0	0
Strictosidine (7c)	1—10	0	0	0	0
1.3 Quaternary monomeric alkaloids					
4,21-Dehydro-geissoschizine (8)	1—10—25	++	0	0	++
Strychnoxanthine (9)	1—10—25	0	0	0	+
N_b-Methyl-harmalane (6e)	1—10	0	0	+	0
Methylantirhine (10)	1—10—25	0	0	+	0
Serpentine Fluka (11)	1—10—25	0	0	0	0
Macusine B (12)	1—10—25	0	0	0	0
Diploceline (13)	1—10—25	0	0	0	0
Malindine (14)	1—10—25	0	0	0	0
Mavacurine (15)	1—10—25	0	0	0	0
1.4 Indole-indoline quaternary dimer					
Afrocurarine (16)	1—10—25	0	0	+	0
2. Oxindole monomeric alkaloid					
Gelsemine Fluka (17)	1—10	0	0	0	0
3. Indoline alkaloids					
3.1. *Tertiary monomers*					
Strychnobrasiline (18)	1—10	0	0	0	0
O-Methyl-N_a-acetyl-strychnosplendine (19)	1—10	0	0	0	0
Holstiline (20)	1—10	0	0	0	0
Strychnine derivatives					
Strychnine (21a)	1—10	0	0	0	0
α-Colubrine Hoffmann (21b)	1—10	0	0	0	0
Vomicine (22a)	1—10	0	0	0	0
19,20α-Epoxy-novacine (22b)	1—10	0	0	0	0
15-Hydroxy-19,20α-epoxy-novacine (22c)	1—10	0	0	0	0
Brucine Serva (21c)	1—10	0	0	0	0
Icajine (22d)	1—10	0	0	0	0

TABLE 1 (*continued*)

Name	Concentration (μg/ml)	B16	2002	L1210	HeLa
3.2. Tertiary dimer					
Bisnordihydrotoxiferine (23a)	1—10	0	0	0	0
3.3. Quaternary monomer					
Fluorocurarine (24)	1—10—25	0	0	0	0
3.4. Quaternary dimer					
Alloferine Roche (23b)	1—10—25	0	0	0	0

analyse rapidly and compare cytotoxic activities of various chemical compounds under the same experimental conditions. This test does not need expensive equipment and offers reproducible results.

We plan to do further experiments with more detailed cytological analysis for the most active compounds and eventually to prepare new semi-synthetic compounds in order to investigate further structure-activity relationships.

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