Evaluation of a selection of Coumarin And Pyrazolotriazine-Derived Compounds as potential modulators of Adenylyl Cyclase Subtype I

Gonzalo Amezquita C, Tarsis F. Brust, Dr. Val J. Watts, Mario F. Guerrero

ABSTRACT

The adenylyl cyclase subtype I (AC1) has shown to be involved in several physiological processes and could be a novel target for the treatment of chronic pain. Therefore, the development of drugs that modulate its activity currently has become a topic of great importance. In the present work, the evaluation of a set of coumarin and pyrazolotriazine-derived compounds was carried out by running a HTRF-based cAMP accumulation assay. A selection of pyrazolotriazines exerted inhibition for the ionophore A23187-stimulated cAMP production on HEK-hAC1 cells, unlike the coumarins which did not show any activity.

***Keywords*— Adenylyl Cyclase Subtype I, Coumarins, HTRF-based cAMP accumulation assay,pyrazolotriazines.**

I. Introduction

Adenylyl cyclases are ATP-pyrophosphate lyases which work like effector proteins that convert ATP into cAMP. Most of the 9 transmembrane subtypes and the soluble subtype have shown to be involved in different physiological processes and, in particular for AC1, roles in memory, pain response and development of drug dependence have been demonstrated in knockout mice studies.

For instance, AC1 (-/-) mice have affected LTP (Long Term Potentiation), which stands for memory disturbances; they do not show response in the intermediate and late phase of the acute muscle pain, and for AC1/8 (-/-) mice the Conditioned Place Preference for the compartment where morphine was administrated is reduced [1].

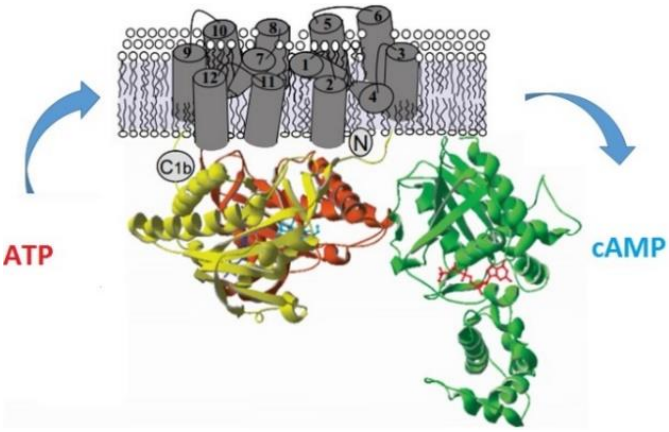

Fig. 1. Crystal structure of AC showing the active site, Forskolin binding site and Gαs protein (Taken and modified from [1]).

In consequence, the development of AC modulators would open the door to further research around the inhibition of these enzymes and the structural requirements of potential drugs that target them.

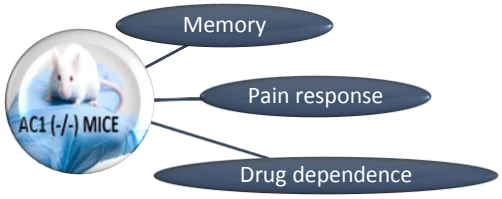

Fig. 2.Physiological processes in which AC1 has a determinant role according to KO mice studies.

Some pyrazolotriazine and coumarin-derived compounds have been patented as drugs for treating CNS (Central Nervous System) diseases. Whereas pyrazolotriazines are synthetically obtained, coumarins are found as secondary metabolites in plants, and due to the mentioned interesting roles in drug development, a selection of these compounds was tested in this work (Annex I).

First Author: gamezquitac@unal.edu.co, Pharmaceutical Chemist, Universidad Nacional de Colombia-Sede Bogotá

Second Author: tbrust@scripps.edu, postdoctoral fellow in the Departments of Molecular Therapeutics and Neuroscience at The Scripps Research Institute - Florida

Third Author: wattsv@purdue.edu , Professor, Molecular Pharmacology and Medicinal Chemistry Department, College of Pharmacy, Purdue University

FourthAuthor: mfguerrerop@unal.edu.co, Professor, Departamento de Farmacia, Universidad Nacional de Colombia

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II. METHODS

Cryopreserved HEK-hAC1 cells are washed and resuspended in OptiMEM media to be plated in a low or regular volume 384-well plate. They are incubated at 37°C and 5% CO2 during 1 hour. Posteriorly, they are treated with the inhibitors solution, which is followed by 30 min of incubation at room temperature (RT). In third place, the addition of the stimulants solution (either case: Forskolin, A23187 and/or Forskolin + A23187) is done and the cells are allowed to incubate during one hour at RT.

After the incubation of the cells with the stimulants, cAMP production by HEK-hAC1 cells is measured using a HTRF (Homogeneous Time Resolved Fluorescence)-based technique. For doing so, the cAMP-D2 and the cryptate conjugate are added and the plate is incubated during 1 hour at RT (Fig.3).

Finally, the fluorescence at 620 nm and 665 nm is determined using a Synergy4 (BioTek, Winooski, VT) fluorescence plate reader (excitation filter: 330/80 nm and emission filters: 620/10nm and 665/8nm). The 665nm/620nm fluorescence ratios are analyzed usingGraphPad Prism 6 (GraphPad Software, La Jolla, CA) to obtain the cAMP concentrations by means of a standard curve. The positive control for inhibition of the A23187 stimulated cAMP production was the reference compound VW-400.

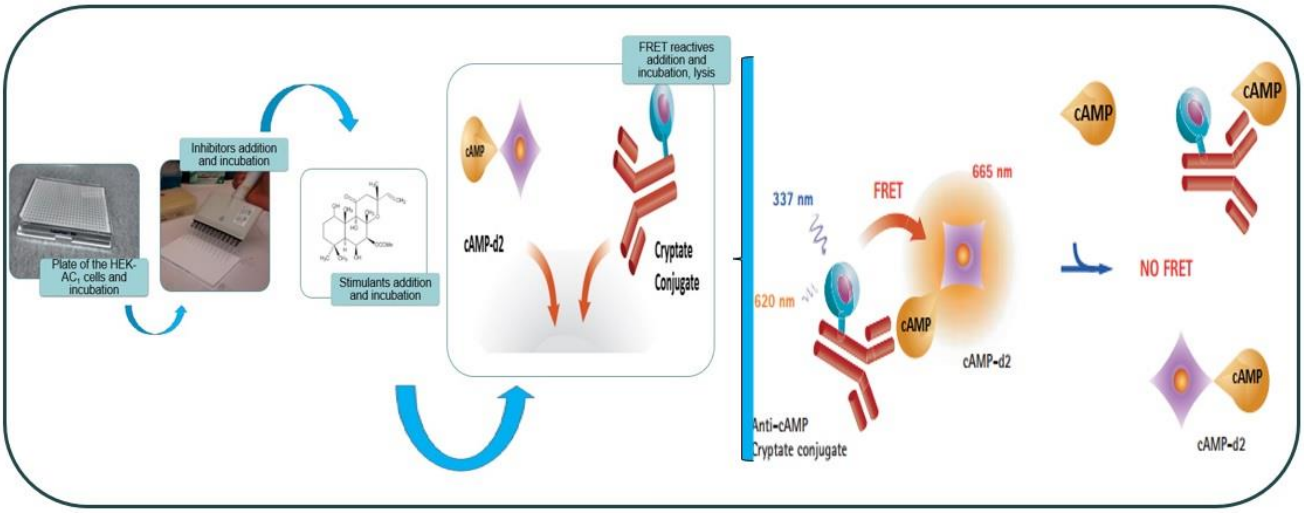


Fig. 3.cAMP accumulation assay: First, cells are plated in 384-well low or regular volume plate. Second, they are incubated with the compounds to test for 30 min which is followed by the Forskolin and/or A23187 ionophore addition to stimulate cAMP production. Finally, cells are lysed and the total cAMP is quantified using the anti-cAMPcryptate conjugate. Depending on the amount of cAMP that cells produce, the antibody binds to the labelledcAMP in a higher or lower ratio which is reflected by the fluorescence at 665 nm. (Modified from Cyclic AMP cell-based assay dynamic 2, Cisbio International [2])

III. RESULTS

Control (+) W400	
Basal	
FSK+A23187	

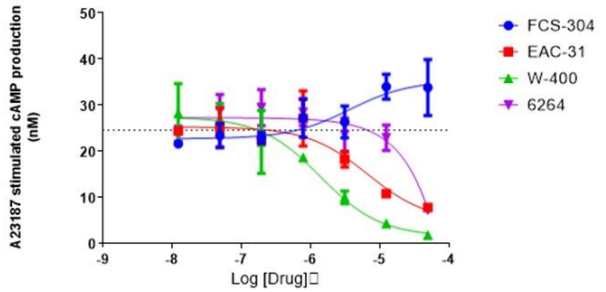
	30µM		3µM		30µM		3µM		30µM		3µM		30µM		3µM		30µM		3µM		30µM		3µM	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	FCS-005		FCS-005		FCS-101		FCS-101		FCS-103		FCS-103		FCS-104		FCS-104		FCS-106		FCS-106		FCS-107		FCS-107	
B	FCS-110		FCS-110		FCS-301		FCS-301		FCS-303		FCS-303		FCS-304		FCS-304		FCS-404		FCS-404		4-OHMeC		4-OHMeC	
C	MH4a		MH4a		EAC-89		EAC-89		EAC-25		EAC-25		EAC-21		EAC-21		EAC-33		EAC-33		EAC-31		EAC-31	
D	W400		W400		Basal		Stimulants		Stimulants		Basal													

Table 1. Selection of coumarin and pyrazolotriazine-derived compounds tested in the initial screening.

	30µM		3µM		30µM		3µM		30µM		3µM		30µM		3µM		30µM		3µM		30µM		3µM	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	40	32	1.7	26	63	59	27	33	45	35	41	32	24	28	33	16	4.2	-9	14	15	40	40	24	8.6
B	31	27	22	2.5	51	50	22	24	32	30	20	22	105	-21	105	-37	105	-19	11	-11	51	47	15	8
C	20	23	15	15	48	33	30	25	78	87	33	29	73	76	46	40	58	57	0.8	27	83	81	45	56
D	101	99	56	45	103	102	-6	-1	-12	18	105													

Table 2. Inhibition percent on the A23187+Forskolin stimulated cAMP production by the selection of coumarin and pyrazolotriazine-derived compounds.

First of all, an initial screening was run with 3 µM and 30 µM drugs solutions on HEK-hAC1 cells. Inhibition on Forskolin + A23187 stimulated cAMP production was found for three pyrazolotriazine-derived compounds (EAC-21, EAC-25 and EAC-31). In addition, for a couple of coumarin-derived compounds (FCS-304 and FCS-404), very different duplicates were gotten as it is shown in the Table 2.



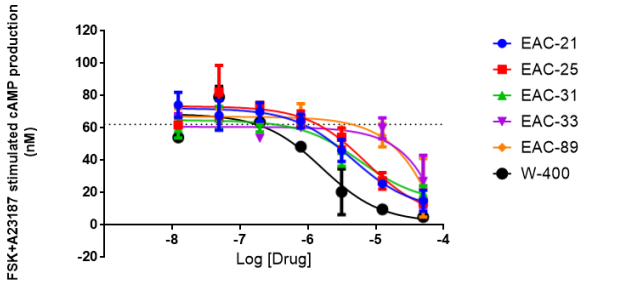
	FCS-304	EAC-31	W-400	6264
log(inhibitor) vs. response (three parameters				Ambiguous
Best-fit values				
Bottom	35.20	4.587	1.387	~ -1535
Top	22.59	25.17	27.32	27.17
LogIC50	-5.439	-5.169	-5.854	~ -2.414
IC50	3.639e-006	6.778e-006	1.399e-006	~ 0.003858
Span	-12.62	20.58	25.93	~ 1562

Average [cAMP] (nM)	
Basal	3.048767143
Stim.	24.47325

Fig. 4. Dose-response curves for one of each type of compound using OptiMEM dilutions for the inhibition of A23187 stimulated cAMP production.

In second place, one pyrazolotriazine and one coumarin were chosen to carry out the assay under regular conditions (5,0µL cells (2500cells/well); 5,5 µL 100 µM Drug solutions; 2,5 µL A23187 12 µM (4X), and 6,5 µL cAMP-D2 or Cryptate conjugate (Total V: 26 µL)) (Fig. 4).

Due to the fact that EAC-31 exerted inhibition on A23187 stimulated cAMP production in this last assay, the whole set of available pyrazolotriazines was tested for inhibitory activity on Forskolin + A23187 stimulated cAMP production. Unlike pyrazolotriazines, coumarin-derived compounds were not tested further.



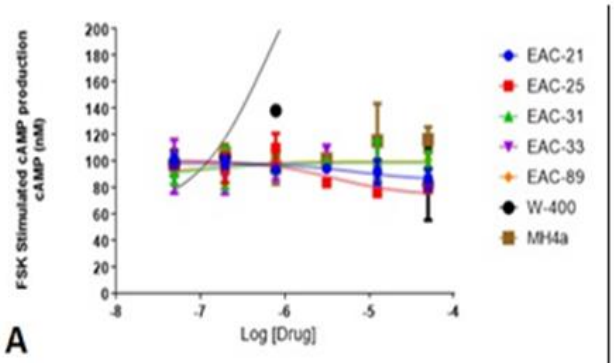
	EAC-21	EAC-25	EAC-31	EAC-33	EAC-89	W-400
log(inhibitor) vs. response (three parameters)				Ambiguous		
Best-fit values						
Bottom	9.264	4.211	13.60	~ -47634	-154.4	1.477
Top	72.06	73.33	64.70	60.54	66.86	68.57
LogIC50	-5.346	-5.146	-5.222	~ -1.132	-3.695	-5.790
IC50	4.513e-006	7.152e-006	6.000e-006	~ 0.07378	0.0002019	1.621e-006
Span	62.80	69.12	51.10	~ 47694	221.2	67.09

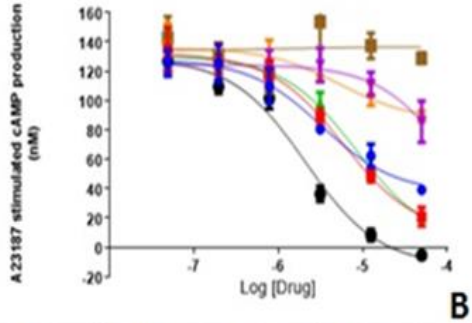
CONTROL	Average
Basal	4.6591954
FSK+A23187	62.087435

Fig.5. Dose-response curves for the pyrazolotriazine-derived compounds for the inhibition of Forskolin+A23187 stimulated cAMP production.

EAC-21, EAC-25 and EAC-31 showed to exert inhibition on the Forskolin + A23187 stimulated cAMP production (Fig. 5).

Finally, in order to clarify the way in which these compounds were acting, Dose-Response Curves were obtained using only A23187 orForskolin. According to the results, neither of thepyrazolotriazinesinhibits the Forskolin stimulated cAMP production, but EAC-21, EAC-25, and EAC-31 (Table 4) do inhibit A23187 stimulated cAMP production as it is shown in the Fig.6A and 6B respectively.





	EAC-21	EAC-25	EAC-31	EAC-33	EAC-89	W-400
Bottom	37.17	8.829	2.663	-17.74	85.93	-12.63
Top	128.1	132.2	130.1	124.4	135.8	128.7
LogIC50	-5.485	-5.235	-5.078	-3.878	-5.264	-5.679
IC50	3.272e-006	5.823e-006	8.353e-006	0.0001326	5.445e-006	2.095e-006
Span	90.90	123.4	127.4	142.2	49.92	141.3

Fig. 6. Dose-response curves for the pyrazolotriazine-derived compounds for the inhibition of Forskolin (Left Side) or A23187 (Right side) stimulated cAMP production.

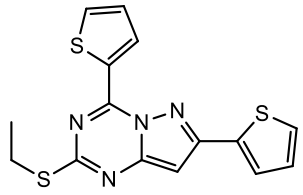
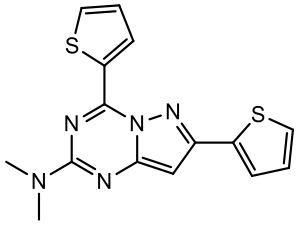
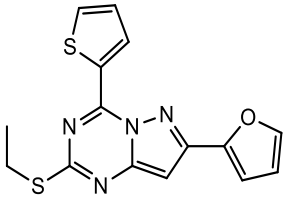
Pyrazolotriazines		
EAC-21	EAC-25	EAC-31
		
2-(ethylsulfanyl)-4,7-dithiophen-2-ylpyrazolo[1,5-a][1,3,5]triazine	N,N-dimethyl-4,7-dithiophen-2-ylpyrazolo[1,5-a][1,3,5]triazin-2-amine	2-(ethylsulfanyl)-4-thiophen-2-yl-7-furan-2-ylpyrazolo[1,5-a][1,3,5]triazine

Table 4. Structure of the pyrazolotriazines that exerted inhibition on A23187-stimulated cAMP production

IV. DISCUSSION

The discovery of AC1 inhibitors has a lot of importance because of the interesting roles this enzyme has shown to be related with. In this work, it was found a set of pyrazolotriazines have potential as modulators of the cAMP production exerted by this protein.

Because of the inhibition EAC-21, EAC-25 and EAC-31 exerted during the experiments carried out, they are discarded as direct AC1 inhibitors. The exerted activity on the A23187 stimulated cAMP production is concordant with Calcium-calmodulin pathway inhibitors instead of Adenylyl cyclase subtype I inhibitors, which is the kind of compounds that was desired to find in this particular study.

Nevertheless, further research could be done to discover and confirm the actual mechanism of action these compounds have. In addition, it would be interesting to determine how different they are in comparison with VW-400, which inhibits calmodulin and how the differences between their structures affect the IC_{50} values. This last would make easier to define Structure-activity relationships.

V. CONCLUSIONS

From the total set of compounds only the pyrazolotriazines EAC-21, EAC-31 and EAC-33 exerted inhibition on A23187 stimulated cAMP production which makes them Calcium-calmodulin pathway inhibitors. Their mechanism of action could be confirmed by means of additional assays where calmodulin activity can be measured.

In addition, because this pyrazolotriazines seems to share the same mechanism of action of the reference compound VW-400, additional studies regarding the structure-activity relationship between them could be done.

ANNEXES

ANNEX I: Structures of the tested coumarin and pyrazolotriazine-derived compounds

ACKNOWLEDGEMENTS

I want to thank Dr. Val J. Watts, Monica Soto Velasquez, Trevor Doyle, Dr. Jason Conley, and specially to Dr. TarsisBrust who taught me to work in Dr. Watts´ lab. In the same way, I feel very grateful with Dr. Mario Francisco Guerrero Pabon who supported me during this project.

Thanks to Purdue University and Universidad Nacional de Colombiafor their support, and to my family for its love.

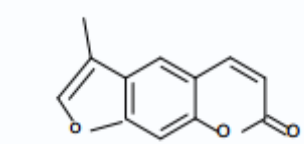
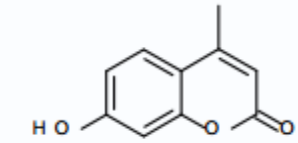
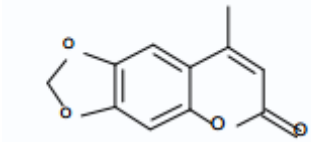
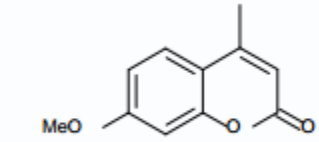
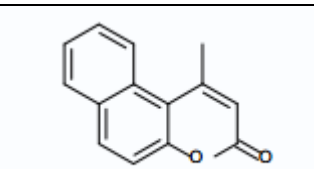
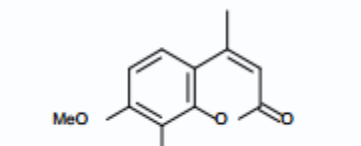
REFERENCES

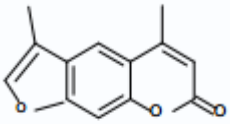
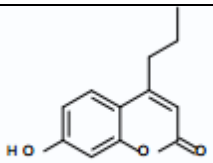
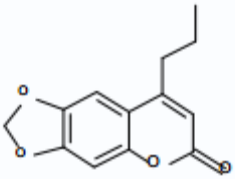
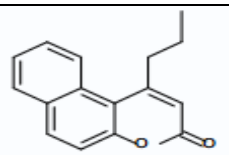
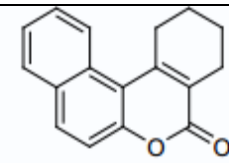
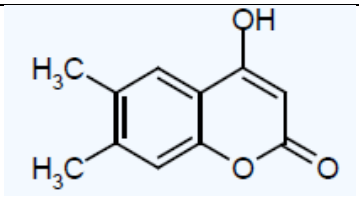
[1] R. Sadana, C. W. Dessauer ,Physiological roles for G-protein regulated adenylyl cyclase isoforms: insights from knockout and overexpression studies

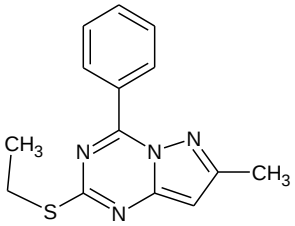
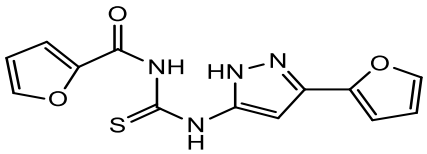
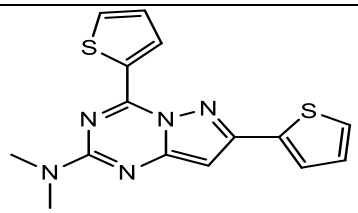
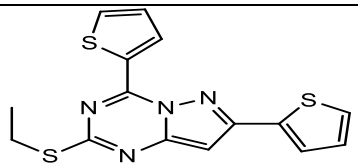
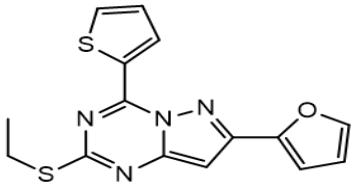
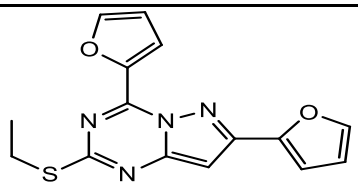
[2]. Cisbio International, HTRF® package insert:cAMP dynamic 2 20,000 tests, France 2009

ANNEX I

Structures of the tested coumarin and pyrazolotriazine-derived compounds

Name	Kind of molecule and structure	Molecular weight (g/mole)
Coumarins		
FCS-005	 3-metil-7*H*-furo[3,2-*g*]cromen-7-ona	200
FCS-101	 7-hidroxi-4-metil-2*H*-cromen-2-ona	176
FCS-103	 8-metil-6*H*-[1,3]dioxolo[4,5-*g*] chromen-6-one	204
FCS-104	 7-metoxi-4-metil-2*H*-cromen-2-ona	190
FCS-106	 1-metil-3*H*-benzo[*f*]cromen-3-ona	210
FCS-107	 7,8-dimetoxi-4-metil-2*H*-cromen-2-ona	220

FCS-110	 3,5-dimetil-7*H*-furo[3,2-*g*]cromen-7-ona	214
FCS-301	 7-hidroxi-4-propil-2*H*-cromen-2-ona	204
FCS-303	 8-propil-6*H*-[1,3]dioxolo[4,5-*g*]cromen-6-ona	232
FCS-304	 1-propil-3*H*-benzo[*f*]chromen-3-one	238
FCS-404	 1,2,3,4-tetrahidro-dibenzo[*c*,*f*]cromen-5(2*H*)-ona	250
4-OHMeC	 4-Hydroxy-6,7-dimethylcoumarin	176

Name	Kind of molecule and structure	Molecular weight (g/mole)
pyrazolo [1,5-a][1,3,5] triazines		
MH4a	 2-(ethylsulfanyl)-7-methyl-4-phenylpyrazolo[1,5-*a*][1,3,5]triazine	270,353
EAC-89	 *N*-[(3-furan-2-yl-1*H*-pyrazol-5-yl)carbamothioyl]furan-2-carboxamide	302,308
EAC-25	 *N*,*N*-dimethyl-4,7-dithiophen-2-ylpyrazolo[1,5-*a*][1,3,5]triazin-2-amine	327,427
EAC-21	 2-(ethylsulfanyl)-4,7-dithiophen-2-ylpyrazolo[1,5-*a*][1,3,5]triazine	344,478
EAC-31	 2-(ethylsulfanyl)-4-thiophen-2-yl-7-furan-2-ylpyrazolo[1,5-a][1,3,5]triazine	320,900
EAC-33	 2-(ethylsulfanyl)-4,7-difuran-2-ylpyrazolo[1,5-*a*][1,3,5]triazine	312,346