PROJECT REPORT

PROJECT TITLE: **Novel scaffolds of protein kinase inhibitors**
PROJECT NUMBER: **62p9**
APPLICANT (CZ): **Vladimír Kryštof**
APPLICANT (AT): **Wolfgang Holzer**

1. Travels from CZ to AT

Date: 27.9.-30.9. 2012
Name: Vladimír Kryštof, Ph.D. (scientist)
Description: discussion about the obtained results (biological screening performed in the Czech lab), discussion about structure-activity relationships, suggestions for direction of further synthetic modification of the desired compounds, preparation of next short term visits

Date: 21.-23.11.2012
Name: Marek Zatloukal, Ph.D. (scientist)
Description: Training NMR techniques which are suitable for the structure determination of oligopeptides. He performed some key NMR experiments with the use of Bruker Avance 500 NMR spectrometer. He also discussed the experiences with microwave chemistry approaches for the synthesis of natural compounds and they also performed a few reactions using high-performance micro-wave reactor (*Synthos 3000*).

Date: 21.-23.11.2012
Name: Tomáš Gucký, Ph.D. (scientist)
Description: He performed some key NMR experiments with the use of Bruker Avance 500 NMR spectrometer. He also discussed the experiences with microwave chemistry approaches for the synthesis of natural compounds and they also performed a few reactions using high-performance micro-wave reactor (*Synthos 3000*).

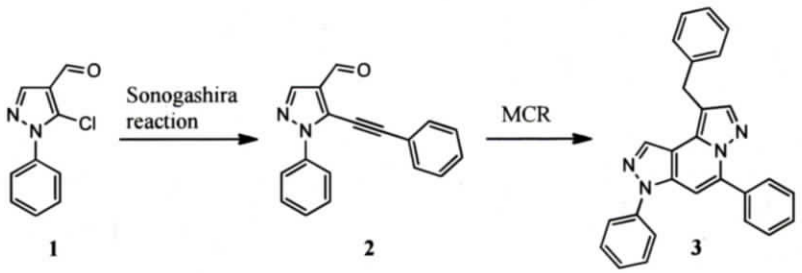
2. Travels from AT to CZ

Date: 14.-18.10.2012
Name: Gyte Vilkauskaite (scientist)
Description: a short course of flow chemistry using apparatus X-Cube continuous-flow heterogenous catalyst/reagent reactor (ThalesNano), a series of Suzuki coupling reactions with various nitrogen containing heterocycles, a short training of flash column chromatography using reverse phase using the *Versa Flash* purification station (*Supelco*), cell culture techniques and biochemical assays, analysis and discussion about results of assays perfored with compounds prepared by G. Vilkauskaite

3. Scientific outcomes and future significance

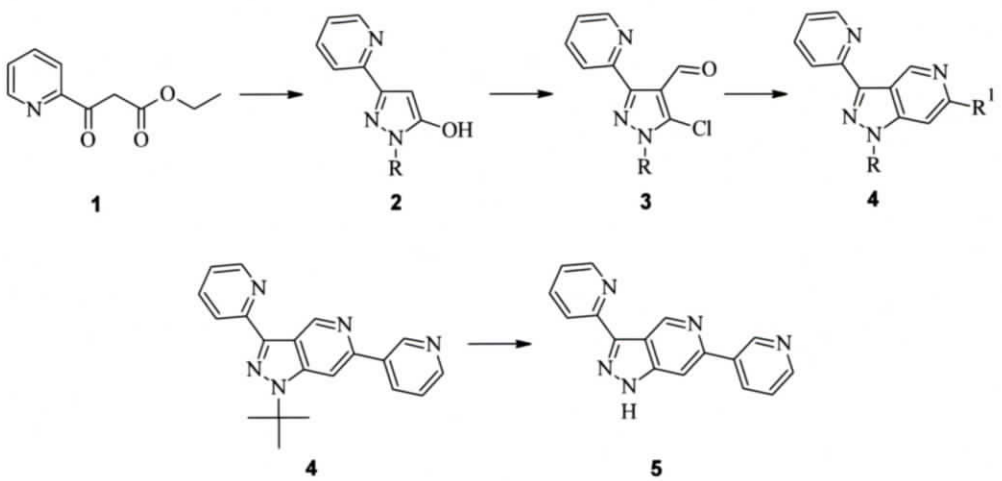
It was planned to synthesize compounds containing following heterocyclic cores: 3*H*-dipyrazolo[1,5-*a*:4',3'-*c*]pyridine, 2*H*-pyrazolo[4,3-*c*]pyridine and dihydrodipyrazolo[3,4-*b*:3',4'-*d*]pyridine-4(1*H*)-one, expecting them to serve as kinase inhibitors.
First target compound, namely 9-benzyl-3,5-diphenyl-3*H*-dipyrazolo[1,5-*a*:4',3'-*c*]pyridine (**3**) was obtained starting from 5-chloro-1-phenyl-1*H*-pyrazole-4-carbaldehyde (**1**). The latter was reacted with phenylacetylene under palladium-catalyzed Sonogashira cross-coupling reaction conditions affording 1-phenyl-5-(phenylethynyl)-1*H*-pyrazole-4-carbaldehyde (**2**). The aldehyde **2** was successfully transformed into the desired substituted tricycle **3** *via*

multicomponent reaction (MCR) with *p*-toluenesulfonyl hydrazide, AgOTf and 3-phenylpropanal (Scheme 1).



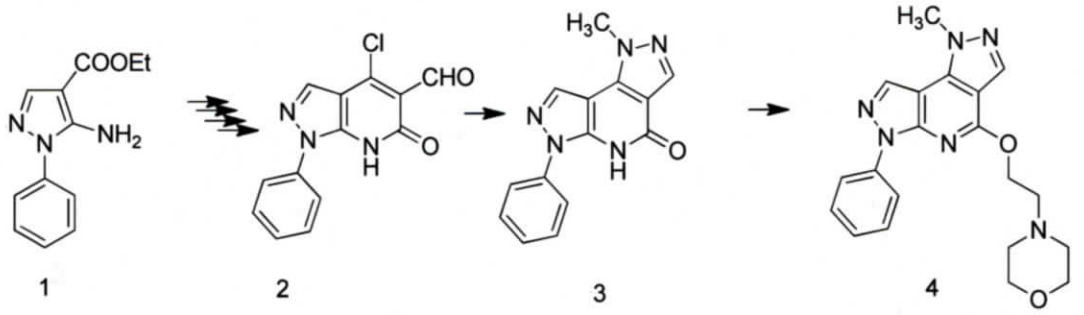
Scheme 1

The synthesis of the second target product 3-(2-pyridinyl)-6-(3-pyridinyl)-1*H*-pyrazolo[4,3-*c*]pyridine (**5**) is outlined below in Scheme 2. Thus, β-keto ester **1** was reacted with substituted hydrazines to obtain corresponding pyrazolones **2**, containing different protecting groups (R = methyl, benzyl, *tert*-butyl). The latter pyrazolones were modified through Vilsmeier-Haak reactions leading to 5-chloropyrazole-4-carbaldehydes of type **3**. Various pyrazole-4-carbaldehydes **3** smoothly underwent Sonogashira cross-coupling reaction and subsequent cyclization affording the corresponding pyrazolo[4,3-*c*]pyridines **4**. Initial attempts to remove methyl or benzyl protecting groups were unsuccessful. The desired target product **5** finally was obtained after successful cleavage of *tert*-butyl protecting group (Scheme 2). Compounds of type **4** (R = methyl, benzyl, R^1 = 3-pyridinyl) were submitted for the biological tests to be carried out in Olomouc as well.



Scheme 2

As the third type of target compounds were envisaged derivatives of dihydrodipyrazolo[3,4-*b*:3',4'-*d*]pyridine-4(1*H*)-one. These compounds were prepared in the following way (Scheme 3): Starting from commercially available aminopyrazole ester **1** the chloroaldehyde **2** was obtained in four reaction steps. Ring closure to assemble the second pyrazole moiety employing methylhydrazine afforded tricycle **3**, subsequent *O*-alkylation with appropriate morpholinoethyl chloride finally led to target compound **4** (= WH-EM4 = 1-methyl-4-[2-(4-morpholinyl)ethoxy]-6-phenyl-1,6-dihydrodipyrazolo[3,4-*b*:3',4'-*d*]pyridine).



Scheme 3

It should be emphasized that the structures of all compounds obtained in the course of the various synthetic procedures were unambiguously confirmed by the combined application of different one- and two-dimensional NMR spectroscopic experiments observing not only ^{1}H, but also ^{13}C and ^{15}N nuclei. Moreover, identity and purity of all compounds were additionally checked by mass spectrometry and elemental analyses (C,H,N). In summary, all synthetic goals of the regarding project could be achieved, however, especially for compound **5** of Scheme 2, with a remarkably larger effort than initially expected.

3.2. Biological activity

All prepared compounds have been tested in a series of assays well-established in the laboratory of Czech applicant. First of all, the compounds have been analyzed in kinase inhibition assays with CDK1/cyclin B, CDK2/cyclin E, CDK9/cyclin T and c-Abl. Cytotoxic activity of tested compound has been quantified by a Calcein AM assay in a microtitre plate. Antiproliferative activity of the compounds has been studied by flow cytometry, detecting simultaneously intercalation of propidium iodide and incorporation of 5-bromo-2'-deoxyuridine (BrdU). Furthermore, proapoptotic potential of CDK inhibitors in cancer cell lines has been studied by flow cytometry (accumulation of sub-G1 population), immunoblotting (fragmentation of PARP) and biochemically (assay of caspase 3).

In sum, 36 compounds were screened for biological activities. Of these, 6 compounds have been prepared during this project, the rest (30 compounds) have been prepared in the Austrian lab in earlier. Five compounds of these (AH31, AH31R, VW11, VW16, WH-EM04) showed significant antiproliferative/cytotoxic activities in K562 and MCF7 cell lines derived from chronic myeloid leukemia and breast carcinoma, respectively. IC_{50} values ranged from 14 to 90 μM. These compounds blocked proliferation of tested cells and induced cell death with typical marks of apoptosis (chromatin condensation, cleavage of PARP, activation of caspase 3). In addition, two compounds (WB25 and PS27) have been found to inhibit activity of recombinant protein kinases CDK2 and related CRK3, both in micromolar range. Based on these preliminary results, we will try to modify the structures to describe basic relationships between structure and activity of the active compounds. Furthermore, we will also study mechanism of antiproliferative/cytotoxic activities of the active compounds.

Significance of the Project

This project helped to establish a collaboration between experienced groups of Austrian organic chemists and Czech biochemists. The project proved the synergy between the two research groups that was based on synthesis of novel heterocyclic compounds, their detailed chemical characterization at the Austrian side and then testing of their biological activities at the Czech side. The established collaboration will bring a benefit to both participating parties. We expect that the results obtained during the project will be the presented at scientific meetings and published in peer-reviewed scientific journals.

I declare hereby that the project report is correct.

24. 1. 2013

Doc. RNDr. Vladimír Kryštof, Ph.D.

ao. Univ.-Prof. Dr. Wolfgang Holzer

ZÁVĚREČNÉ VYÚČTOVÁNÍ

NÁZEV PROJEKTU: **Novel scaffolds of protein kinase inhibitors**
ČÍSLO PROJEKTU: **62p9**
ŘEŠITEL (CZ): **Vladimír Kryštof**

Náklady – celkový přehled

Uvedená tabulka obsahuje informace o částce přidělené českému řešiteli projektu a částkách čerpaných v ČR.

	Přidělené na rok 2012	Čerpané v roce 2012
Cestovné, ubytování	46.000 CZK	11.285 CZK
Věcné náklady	36.000 CZK	36.000 CZK
Celkové náklady	82.000 CZK	47.285 CZK
Nespotřebované náklady	-	34.715 CZK

Náklady – jednotlivě

Spotřeba materiálu. Veškeré přidělené prostředky byly použity pro pořízení chemikálií a laboratorního materiálu nutného pro řešení projektu (radioaktivně značené ATP, plasty pro tkáňová kultury, protilátka PARP).

Cestovné českých účastníků do Rakouska.

Vladimír Kryštof (27.9.-30.9. 2012)	1.063 Kč	JÍZDNÉ ČD (Olomouc-Wien)
Marek Zatloukal (21.-23.11.2012)	1.461 Kč	JÍZDNÉ ČD (Olomouc-Wien)
Tomáš Gucký (21.-23.11.2012)	1.461 Kč	JÍZDNÉ ČD (Olomouc-Wien)

Pobytové náklady pro účastníky projektů z Rakouska na českém území
ubytování G. Vilkauskaite (14.-18.10.2012) 4.800 Kč
4 noci, jednotková sazba 1200 Kč

Stravné a kapesné pro účastníky z Rakouska v ČR
stravné G. Vilkauskaite (14.-18.10.2012) 2.500 Kč
5 dní, jednotková sazba 500 Kč

Nespotřebované náklady
Nespotřebovaná částka bude vrácena poskytovateli. Důvodem je to, že rakouský spoluřešitel neměl vhodného kandidáta pro vyslání do laboratoře v ČR.

Univerzita Palackého
Výkaz jednotlivých položek na prvcích SPP

Objekt:

Datum účtování: -

Pd	PrÚč	Prvek SPP	VedlPřÚč 1	Nákl.druh	Popis nákl.druhu	Dat.účt.	Hodn./MěnO	MěObj	Označení	Objekt	ČRefDokl
10	31	133104011	ZAK 10	501100	Spotřeba materiálu	29.10.2012	4.400,00	CZK	chemikálie	KB227	1310033790
11	31	133104011	ZAK 10	501100	Spotřeba materiálu	07.11.2012	37.718,40	CZK	laboratorní materiál	KB227	1310034884
11	31	133104011	ZAK 10	501100	Spotřeba materiálu	07.11.2012	36.588,00	CZK	laboratorní materiál	KB227	1310035849
11	31	133104011	ZAK 10	501100	Spotřeba materiálu	22.11.2012	36.588,00-	CZK	5431007637		300224109
11	31	133104011	ZAK 10	501100	Spotřeba materiálu	22.11.2012	6.118,40-	CZK	5431007485 část		300224109
12	31	133104011	ZAK 10	501100	Spotřeba materiálu	17.12.2012	36.331,20	CZK	ProQinase GmbH - 1200 EUR	KB228	1310041803
12	31	133104011	ZAK 10	501100	Spotřeba materiálu	31.12.2012	11.470,22-	CZK	1310041803 část		300226803
12	31	133104011	ZAK 10	501100	Spotřeba materiálu	31.12.2012	24.860,98-	CZK	1310041803 část		300226803
*		133104011	ZAK 10	501100			36.000,00				
11	31	133104011	ZAK 10	512200	Cestovné zaměstnanců - zahraniční	27.11.2012	1.063,00	CZK	1310032011		300224403
12	31	133104011	ZAK 10	512200	Cestovné zaměstnanců - zahraniční	11.12.2012	1.500,00	CZK	Dr Zatloukal Rakousko 21,11-23,11		3295832
12	31	133104011	ZAK 10	512200	Cestovné zaměstnanců - zahraniční	12.12.2012	2.648,00	CZK	Zatloukal, Rakousko, 21.11.2012	KB227	1310040991
12	31	133104011	ZAK 10	512200	Cestovné zaměstnanců - zahraniční	17.12.2012	1.500,00	CZK	Dr Gucký Rakousko 21,11		3296964
12	31	133104011	ZAK 10	512200	Cestovné zaměstnanců - zahraniční	20.12.2012	998,00	CZK	Gucký, Rakousko, 21.11.2012	KB227	1310043146
12	31	133104011	ZAK 10	512200	Cestovné zaměstnanců - zahraniční	31.12.2012	998,00-	CZK	1310043146		300228414
12	31	133104011	ZAK 10	512200	Cestovné zaměstnanců - zahraniční	31.12.2012	2.648,00-	CZK	1310040991		300228414
12	31	133104011	ZAK 10	512200	Cestovné zaměstnanců - zahraniční	31.12.2012	39,00-	CZK	3296964		300228414
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*		133104011	ZAK 10	512200			3.985,00				
10	31	133104011	ZAK 10	518102	Ostatní služby - ubytování a stravování	14.10.2012	4.800,00	CZK	ubytování	KB227	1310031289
*		133104011	ZAK 10	518102			4.800,00				
10	31	133104011	ZAK 10	549100	Jiné ostatní náklady	15.10.2012	2.500,00	CZK	Mgr G. Vilkauskaite pobyt náklady		3006897
*		133104011	ZAK 10	549100			2.500,00				
4	31	133104011	ZAK 10	720691	Vnitrovýnosy - přeúčtování dotací	20.04.2012	82.000,00-	CZK	133104011		300209409
12	31	133104011	ZAK 10	720691	Vnitrovýnosy - přeúčtování dotací	31.12.2012	30.991,00	CZK	vratka,133104011		300226804
12	31	133104011	ZAK 10	720691	Vnitrovýnosy - přeúčtování dotací	31.12.2012	3.724,00	CZK	vratka,133104011		300228414
*		133104011	ZAK 10	720691			47.285,00-				
**		133104011	ZAK 10				0,00				
***			ZAK 10				0,00				
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UNIVERZITA PALACKÉHO V OLOMOUCI
PŘÍRODOVĚDECKÁ FAKULTA
EKONOMICKÉ ODDĚLENÍ
17. listopadu 12, 771 46 Olomouc
DIČ: CZ61989592 -1-