SYNTHESIS OF PYRAZOLE DERIVATIVES, WITH BIOLOGICAL ACTIVITY IN SIGNAL TRANSDUCTION THERAPY

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În timpul unei analize a kinazei realizate în cadrul unui proces de sortare a unei cantităţi mari de substanţă, la Nested Chemical Library (NCL^{TM}) s-a descoperit un puternic derivat pirazol kinazo-inhibitor: 1,3-di-tert-butil-N-(4-fluorofenil)-1,6-dihidroimidazo[4,5-c]pirazol-5-amină. Deoarece mostra avea doar 91,3% puritate, analiza biochimică a fost urmată de resintetizarea moleculei respective în puritate de 97%. Analiza biochimică repetată a arătat că substanţa purificată este inactivă. S-a concluzionat că activitatea biologică ar trebui pusă pe seama uneia sau a mai multor impurităţi minore. Spectrul de masă al mostrei chimice originale a prezentat două componente minore. Aceşti imidazo[4,5-c]pirazole-5-yl)-amino derivaţi au fost sintetizaţi în scopuri de validare.

A potent kinase inhibitor pyrazole derivative, 1,3-di-tert-butyl-N-(4-fluorophenyl)-1,6-dihydroimidazo[4,5-c]pyrazole-5-amine, has been found in Nested Chemical Library (NCL^{TM}) during a high throughput screening (HTS) kinase assay. Since the sample had only 91,3% purity, the biochemical assay was followed by the resynthesis of the hit molecule in a purity of 97%. The repeated biochemical assay showed that the purified substance is inactive. It was concluded that the biological activity should be attributed to one or more minor impurities. The mass spectrum of the original chemical sample showed two minor components. These imidazo[4,5-c]pyrazole-5-yl)-amine derivatives have been synthesized for hit validation purposes.

Keywords: kinase inhibitor, heterocyclic molecule, pyrazole derivatives

1. Introduction

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Signal transduction therapy has become a very important area of drug research; the concept of rational drug design has been expanded for a complex process, including pathomechanism-based target selection, target validation, structural biology, structure-activity relationships, pharmacological optimization. The kinases are probably the most important signaling enzymes, the mitogen-activated protein (MAP) kinases are serine or threonine specific protein kinases that respond to extracellular stimuli and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival or apoptosis. [1, 2]

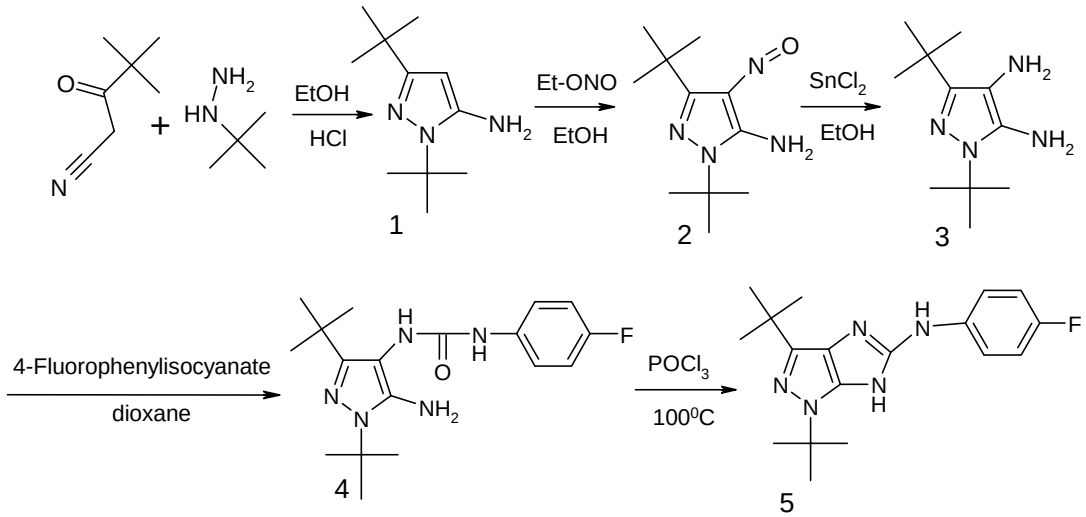
P38 is a recently identified member of the MAP kinase super family. Inhibitors of the MAP kinase p38 provide novel approaches for the treatment of osteoporosis and inflammatory disorders, [1].

Heterocyclic drug like molecules with protein kinases inhibitor activity have emerged as indispensable tools for studying signal transduction. Various pyrazole derivatives were identified as small-molecule inhibitors of p38 kinase.

From the 12,000 membered NCL^{TM} kinase inhibitor library, we have selected hit molecules with significant p38 MAP kinase inhibitory activity. One of these heterocyclic molecules was a derivative of pyrazole: 1,3-di-tert-butyl-N-(4-fluorophenyl)-1,6-dihydroimidazo[4,5-c]pyrazole-5-amine (**5**). Interestingly, only this single molecule showed significant activity among other 25 very similarly structured. Because the original sample had 91,3% purity, this selected compound was reproduced in order to validate the structure and its activity. In conclusion, a good inhibitory activity was expected for this resynthesized molecule on p38 MAP kinase assay.

2. Results and Discussions

The resynthesis of compound **5** has been performed in five steps according to the methods given in a Hungarian patent application, [1].



Scheme 1: Synthesis of 1,3-di-tert-butyl-*N*-(4-fluorophenyl)-1,6-dihydroimidazo [4,5-*c*]pyrazole-5-amine (**5**)

The resynthesis was followed by the biological activity measurement of the compound, in purity of 97%, and it showed that the purified substance was inactive. It was concluded that the biological active component should have been found among minor impurities. The LCMS measurement of the original hit sample showed two minor components with hypothetical structures of 3-tert-butyl-N-(4-fluorophenyl)-1,6-dihydro-imidazo [4,5-c]-pyrazole-5-amine (**7**) and 1,3-di-tert-butyl-N-(4-fluorophenyl)-5-[(4-fluorophenyl)-amino]imidazo[4,5-c]pyrazole-6(1H)-carboxamide (**8**). These compounds have been synthesized for hit validation purposes.

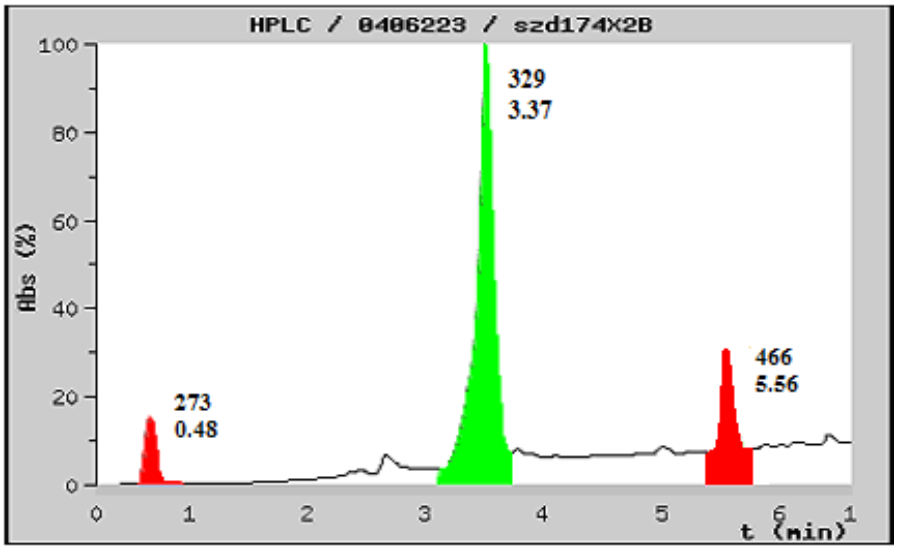
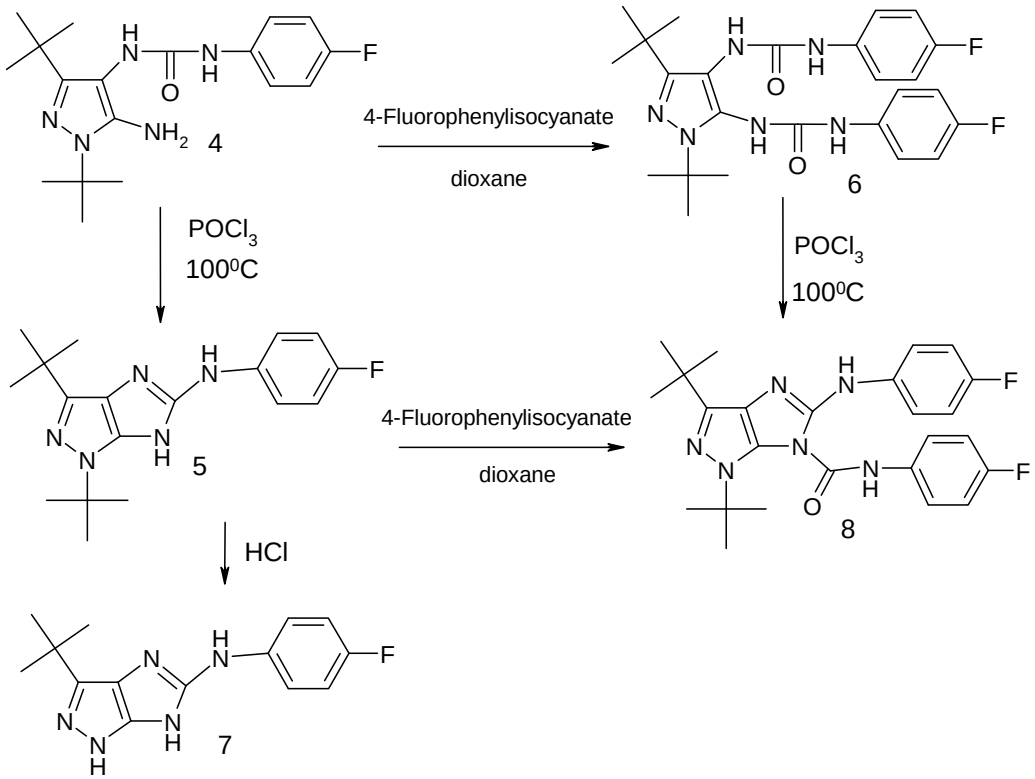


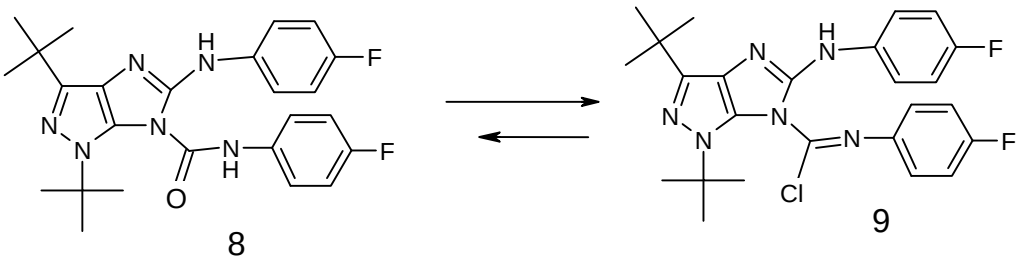
Fig. 1: HPLC chromatogram of the hit sample (1,3-di-tert-butyl-N-(4-fluorophenyl)-1,6-dihydroimidazo[4,5-c]pyrazole-5-amine (**5**),
LC-MS: t_R= 3,37 min; $C_{18}H_{24}N_5F$ (M=329g/mol))

The reactions which might lead to these minor impurities have been studied and it has been found that the conditions of the ringclosure reaction (**4→5**) were strong enough to cleave the tertier-butyl group from the pyrazole nitrogen. Aqueous hydrochloric acid was used in order to complete this reaction and the compound **7** was obtained from **5** in good yield**.** The other impurity, compound **8,** can be derived from **6,** which is a bisacylated side product of the acylation step (**4→5**). 5-10% bisacyl side product has been detected in the reaction mixture during the acylation step (**4→5**), which was easily removed during the purification. The original intermediate **3** might contain bisacyl impurity, which has been transformed to compound **8** during the ringclosure reaction in phosphorous oxichloride. Although compound **8** has been synthesized in a very low yield using the same method, another reaction pathway has been developed, which was much more suitable. In this method, **8** has been directly produced from compound **5**.



Scheme 2: Synthesis of the supposed minor components **7** and **8**

Both minor components were tested against inhibition of p38 MAP kinase and compound **7** and **8** showed no activity. It was supposed that compound **8,** under the reaction conditions of the ringclosure, has been transformed into an imidoylchloride-derivative (**9**), which is a reactive (and probably biologically effective) side product of the reaction. The compound **9** could not be detected in the samples by LCMS, because of its water sensitivity, but this molecule could be responsible for the false positive activity of the hit sample.



Scheme 3: The transformation of compound **8** into imidoylchloride-derivate **9**

All compounds were analyzed by LC-MS and NMR (^{1}H NMR, 300 MHz, DMSO-D_6).

3. Experimental

Synthesis of 2,5-Di-tert-butyl-2H-pyrazole-3-ylamine (1)
The mixture of 4,4-dimethyl-3-oxopentanenitrile (12.5g, 100 mmol) and tert-buthylhydrazine (17.5 g, 198.8 mmol) was refluxed in ethanol (250 ml) with concentrated (cc.) hydrochloric acid (8 ml) for 24 hours. The resulted reaction mixture was made basic with sodium hydroxide and extracted with ethyl acetate. The combined organic extracts were dried over anhydrous sodium sulfate. The solvent was removed on the rotary evaporator to give the desired product as an oil, and it was crystallized from hexane to give the title compound as a white solid (8.76g, Yield: 44.9%).

Synthesis of 2,5-Di-tert-butyl-4-nitroso-2H-pyrazole-3-ylamine (2)
15% Ethyl-nitrite/ethanol (55 ml, 0.58 mmol) was added to 1,3-di-tert-butyl-1*H*-pyrazole-5-amine (2.5g, 12.8mmol) in the presence of cc. hydrochloric acid (1.5 ml) and the mixture was stirred at room temperature for 40 minutes. The resulted reaction mixture was concentrated in vacuum, and crystallized from diethyl ether (1.93g, Yield: 67.2%).

1,3-di-tert-butyl-1H-pyrazole-4,5-diamine (3)
Stannous chloride dihydrate (4.6g, 24.3mmol) in ethanol (4.6ml) and cc. hydrochloric acid (4.6 ml) was added to a solution of 1,3-di-tert-butyl-4-nitroso-1H-pyrazole-5-amine (2.0g, 8.9mmol) in ethanol (20 ml) followed by 1 hour stirring at 70ºC. The reaction mixture was concentrated in vacuum, extracted with ethyl acetate and the combined organic extracts were washed with 1 N sodium hydroxide, then the solvent was removed. The row product was used in the next step without further purification.

1-(5-Amino-1,3-di-tert-butyl-1H-pyrazole-4-yl)-3-(4-fluoro-phenyl)-urea (4)

The 1,3-di-tert-butyl-1*H*-pyrazole-4,5-diamine (1.4g, 6mmol) was dissolved in abs. dioxan (20 ml), then 4-fluorphenyl isocyanate (0.337 ml, 3mmol) was added. The mixture was stirred for 24 hour at room temperature. The precipitated product was suspended in dioxan (20 ml), then filtered and washed with diisopropyl ether. (0.815g, Yield: 35.3%)

1,3-di-tert-butyl-*N*-(4-fluorophenyl)-1,6-dihydroimidazo [4,5-*c*]pyrazole-5-amine (5)

1-(5-Amino-1,3-di-tert-butyl-1H-pyrazole-4-yl)-3-(4-fluoro-phenyl)-urea (0.3g, 0.8 mmol) was dissolved in phosphorus oxychloride (1.5 ml, 16.4mmol) and stirred at 100°C. After 24 hour stirring the reaction mixture was poured into crushed ice with continuous stirring, then it was extracted with ethyl acetate, and it was made basic with solid sodium carbonate. The organic extract was dried over anhydrous sodium sulphate, the solvent was removed and the residue was suspended with hexane, then the solid was filtered off. The product was purified via silica gel chromatography (eluent: 90% chloroform, 10% methanol) to give a white solid. (0.18g, Yield: 64.3%.)

1-{2,5-Di-tert-butyl-4-[3-(4-fluoro-phenyl)-ureido]-2H-pyrazole-3-yl}-3-(4-fluoro-phenyl)-urea (6)

The 1-(5-Amino-1,3-di-tert-butyl-1H-pyrazole-4-yl)-3-(4-fluoro-phenyl)-urea (0.2g, 1 mmol) was dissolved in abs. dioxan (2 ml), followed by addition of 4-fluorphenyl isocyanate (0.113 ml, 1mmol). The reaction mixture was stirred at room temperature for 24 hours. The precipitated product was suspended in dioxan (5 ml), then filtered and washed with diisopropyl ether. (0.108g, Yield: 38.7%)

3-tert-butyl-*N*-(4-fluorophenyl)-1,6-dihydroimidazo[4,5-*c*]pyrazole-5-amine (7)

1,3-Di-tert-butyl-*N*-(4-fluorophenyl)-1,6-dihydroimidazo [4,5-*c*]pyrazole-5-amine (0,05 g, 0,15) was treated with cc. hydrochloric acid (0,5 ml) and the solution was stirred at 80ºC temperature for 48 hours. 1N hydrochloric acid (1 ml) was added to the reaction mixture and it was stirred for additional 24 hours. After that the mixture was made basic with sodium carbonate, and the precipitated product was filtered off. (0.025 g, Yield: 60.38%)

1,3-di-tert-butyl-*N*-(4-fluorophenyl)-5-[(4-fluorophenyl) amino]imidazo[4,5-*c*]pyrazole-6(1*H*)-carboxamide (8)

1-{2,5-Di-tert-butyl-4-[3-(4-fluoro-phenyl)-ureido]-2H-pyrazole-3-yl}-3-(4-fluoro-phenyl)-urea (0.124g, 0.256 mmol) was dissolved in phosphorus oxychloride (2.0 ml, 21.8mmol) and it was stirred at 100°C. After 24 hour the reaction mixture was poured into crushed ice with continuous stirring and it was extracted with ethyl acetate and made basic with 10% sodium carbonate. The organic extract was dried over anhydrous sodium sulphate, the solvent was removed on the rotary evaporator. The residue was solidified under hexane and

the product was filtered, then purified via column chromatography (silica gel, eluent: 95% chloroform, 5% methanol) to give a white solid. (0.006g, Yield: 4%)

1,3-di-tert-butyl-*N*-(4-fluorophenyl)-5-[(4-fluorophenyl) amino]imidazo[4,5-*c*]pyrazole-6(1*H*)-carboxamide (8)

The 1,3-di-tert-butyl-*N*-(4-fluorophenyl)-1,6-dihydroimidazo [4,5-*c*]pyrazole-5-amine (0.064g, 190mmol) was dissolved in abs. piridine (2 ml), followed by addition of 4-fluorphenyl isocyanate (0.210ml, 0,19mmol), then the mixture was stirred at room temperature for 48 hours. The reaction mixture was concentrated in vacuum, and the product crystallized from hexane, then it was filtered off. The product was purified via column chromatography (silica gel, eluent: 95% chloroform, 5% methanol) to give a white solid. (0.015g, Yield: 16.6%)

4. Conclusions

The promising hit molecule, which was identified via HTS kinase assay, showed no activity in biochemical test after its synthesis. Neither the synthesis of the impurities has led to active molecules. It is supposed that the reactive side product is an imidoyl chloride, which could be responsible for the activity in the biochemical assay via chemical reaction with the kinase enzyme.

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