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Pyrrolidinohydroquinazolines—a novel class of CCR3 modulators

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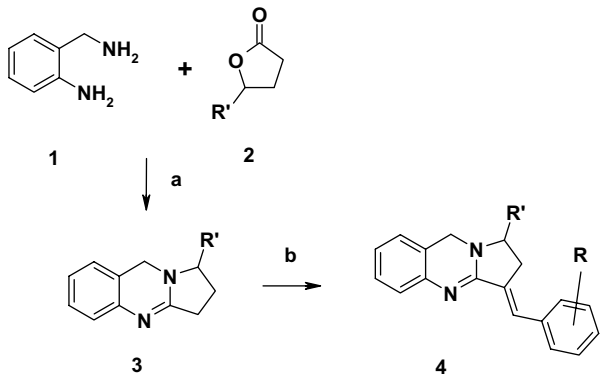
Abstract—A novel class of CCR3 modulators is described. Starting with lead compound **4a** (K_i: 110 nM), which turned out to be an antagonist of eotaxin at the CCR3 receptor, further optimization led to compound **8b** (K_i: 28 nM), which surprisingly proved to be an agonist.

CCR3 is a seven transmembrane, G-protein coupled receptor.[1] Ligands of this receptor include eotaxin-1, -2, -3, RANTES, MCP-3 and MCP-4 among others. Of these ligands, the eotaxins bind to the CCR3 receptor exclusively.[2] CCR3 appears predominantly on eosinophils,[3] cells, which are able to release cytoplasmic components like lipid mediators or cytokines, causing tissue destruction and recruitment of additional inflammatory cells.[4] CCR3 receptors and eotaxin are prevalent in the bronchial mucosa of atopic asthmatics[5] and, together with other evidence, makes it conceivable that CCR3 antagonists have potential as drugs in asthmatic diseases. Information from receptor protein sequences and structures from already known CCR3 antagonists make it putative that a basic centre is beneficial for CCR3 receptor affinity, and companies with known antagonists, for example, LeukoSite,[6] Banyu,[7] Roche,[8] Merck,[9] GlaxoSmithKline,[10] DuPont[11] and Abbott.[12] Bristol–Myers Squibb[13] have published compounds with functional CCR3 agonism. We wish to report here a completely novel and unique structural class of CCR3 modulators.

The target compounds **4**, **8** and **12** were all synthesized by known literature procedures. Condensation of the diamine **1** with γ-butyro lactone **2** gave the intermediate **3**,[14] which was condensed further with benzaldehydes yielding pyrrolidinohydroquinazolines **4**[15] (Scheme 1). The configuration of the exocyclic double bond was controlled in a few cases and was assumed to be *trans* in all analogous compounds. The amino nitriles **5** reacted with lactim ether **6** yielding the known intermediate **7**.[16] After condensation with benzaldehydes the novel alkenes **8** were obtained analogous to compounds **4**, however in lower yields (Scheme 2). The imine nitrogen could be further alkylated by $Me_3O^+BF_4^-$ or BrCN yielding **8f** and **8g**, respectively. Similarly, amino cyano thiophenes **9**, easily available via the Gewald reaction,[17] could be condensed to yield intermediates **10** (Scheme 3). However, **10** reacted with *o*-bromo benzaldehyde to give only the addition products **11**, which required further dehydratation with $POCl_3$ to get the target compounds **12**. The furan, pyridine and imidazole compounds **13**, **14** and **15** (Scheme 4) were synthesized in a similar manner. All substances were characterized by ^1H NMR and mass spectroscopy.



Scheme 1. Synthesis of target compounds **4**. Reagents and conditions: (a) (1) Δ; (2) $POCl_3$; (b) Ar–CHO, Δ.

Keywords: CCR3; Eotaxin; Antagonist; Agonist.
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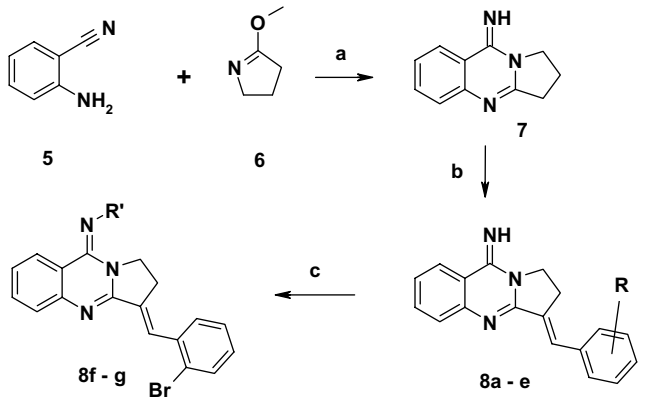


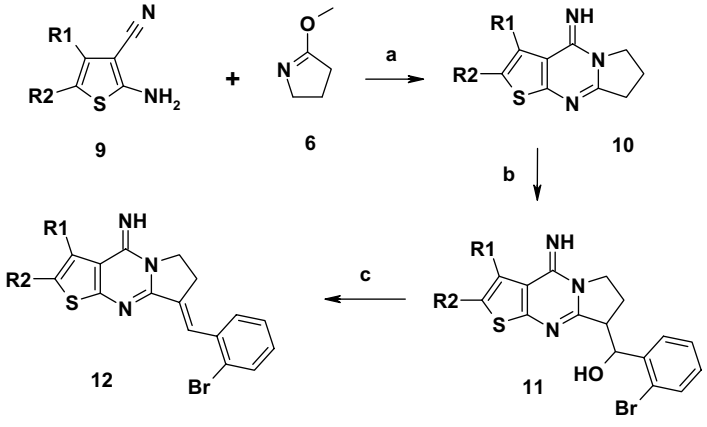
Scheme 2. Synthesis of target compounds **8**. Reagents and conditions: (a) Δ; (b) Ar–CHO, Δ; (c) $Me_3O^+BF_4^-/CH_2Cl_2$ or BrCN/DMF.

CCR3 binding affinities were measured with a binding assay on K562 cell membranes (transfected with human CCR3 receptor) using ^{125}I-eotaxin as radioligand.[18] Tables 1–4 depict the results for the compounds tested using this assay. Compound **4a** was obtained as a screening hit showing a K_i value of 110 nM. Values of analogues previously synthesized within our programme indicated the importance of an *ortho* substituent on the styryl phenyl ring. Consequently, the *ortho* position was further investigated (Table 1, **4a**–**h**). The *o*-bromo compound **4b** showed slightly higher affinity (K_i: 90 nM), but further variations at this position led to poorly active substances (**4c**–**h**). This was surprising, especially for the fluoro or trifluoromethyl substituted compounds. Presumably, there is a specific lipophilic pocket which allows only *ortho* substituents with a defined volume (MR ~ 6–8), and that *ortho* halogen substituent causes the phenyl ring to turn out of the plane. This is consistent with the fact that the *m*- or *p*-chloro compounds were much weaker than **4a** in activity (data not shown). The *o*,*o*-dichloro compound **4i** was also weakly active, further supporting the hypothesis of a specific lipophilic pocket.

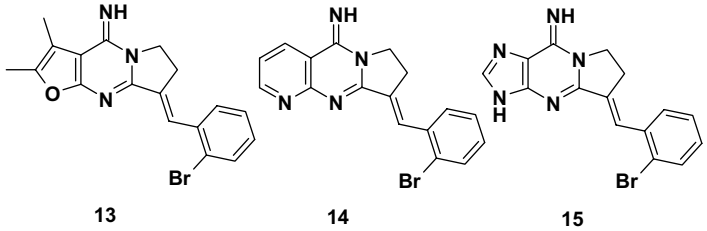
Additional *para* substituents (**4j**–**l**), led to compounds with weak affinity. This was surprising, with even the small fluoro substituent proving detrimental. The *m*,*p*-

Table 1. CCR3 binding affinities of target compounds **4**

Compounds	R	R′	CCR3 binding affinity K_i (nM ± SD)
4a	2-Cl	H	110 ± 44
4b	2-Br	H	90 ± 27
4c	2-F	H	1400 ± 400
4d	2-CF_3	H	1600
4e	2-Et	H	1400 ± 36
4f	2-OEt	H	4500 ± 280
4g	2-Ph	H	2900 ± 770
4h	2-OCF_3	H	3200 ± 660
4i	2,6-Di-Cl	H	1800 ± 360
4j	2-Cl, 4-F	H	930 ± 75
4k	2-Cl, 4-NMe_2	H	1400 ± 230
4l	2,4-Di-Cl	H	1700 ± 380
4m	3,4-Di-Cl	H	720 ± 23
4n	2-Cl	Et	140 ± 40
4o	2-Br	*n*-Bu	250 ± 2.5
4p	2-Br	4-F–Ph	7900 ± 1900



Scheme 3. Synthesis of target compounds **12**. Reagents and conditions: (a) Δ; (b) *o*-Br–Ph–CHO, Δ; (c) $POCl_3$.



Scheme 4. Target compounds **13**–**15**.

Table 2. CCR3 binding affinities of target compounds **8**

Compounds	R	R′	CCR3 binding affinity K_i (nM ± SD)
8a	2-Cl	H	35 ± 18
8b	2-Br	H	28 ± 15
8c	2-I	H	76 ± 6.0
8d	2-Et	H	360 ± 50
8e	4-OMe	H	3900 ± 560
8f	2-Br	Me	310 ± 130
8g	2-Br	CN	6100

Table 3. CCR3 binding affinities of target compounds **12**

Compounds	R1	R2	CCR3 binding affinity K_i (nM ± SD)
12a	H	H	28 ± 10
12b	*c*-Pr	H	290 ± 110
12c	*t*-Bu	H	1200 ± 120
12d	Me	Me	260 ± 85
12e	$-(CH_2)_4-$		350 ± 72

dichloro compound **4m** was also weakly active. The substituent R′ was also varied. An ethyl group at this position showed equal affinity to **4a**, whereas a *n*-butyl chain and a *p*-fluoro-phenyl group were detrimental (**4n**–**p**), indicating that there is only space for a lower alkyl group.

The main polar pharmacophore of compounds **4** is apparently the basic cyclic amidine moiety. Therefore it was postulated that enhancement of this basicity would lead to more active compounds, as in the case of the imine derivatives **8** (Table 2). Indeed, the *o*-chloro and *o*-bromo compounds **8a** and **8b** showed higher affinities (K_i: 35 and 28 nM, respectively). The *o*-iodo derivative **8c** retained some activity (K_i: 76 nM), whereas the *o*-ethyl and *p*-methoxy compounds were weaker. The importance of the basic imine centre was proved by the results of the N-methyl compound **8f** (weaker than **8b** by a factor of 10) and the much less basic N–CN derivative **8g**, which is only marginally active. Further potential for optimization of this part of the molecule was considered unlikely.

Table 4. CCR3 binding affinities of target compounds **13**–**15**

Compounds	CCR3 binding affinity K_i (nM ± SD)
13	270 ± 56
14	820 ± 67
15	830 ± 190

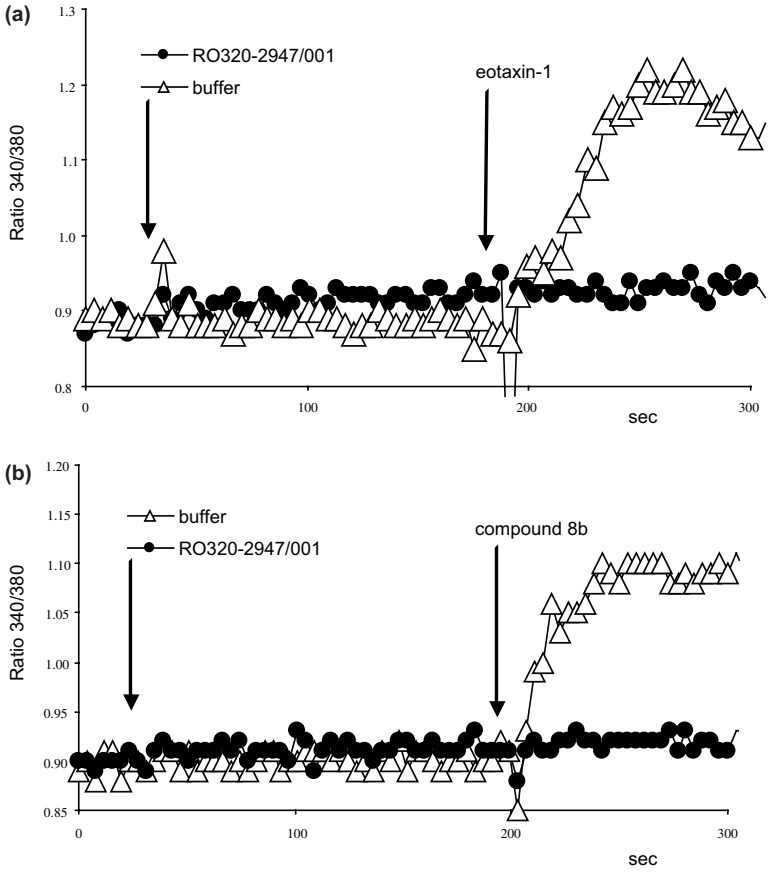


Figure 1. Calcium mobilization of (a) eotaxin-1 and (b) compound **8b**. Ratio of intracellular calcium mobilization in human peripheral eosinophils (FURA-2 loaded cells) in presence of 0.1% BSA. Eosinophils were preincubated with buffer (-△-) and CCR3 antagonist RO320-2947/001 (-●-) 1 μM at the point of the left hand arrow. After 3 min, subsequently all the cells were stimulated with (a) eotaxin-1 (10 nM); (b) compound **8b** (1 μM) at the point of the right hand arrow.

Because 2-amino-3-cyano-thiophenes are readily available as starting materials, thiophenes **12** were well suited to explore the role of alkyl substituents on this part of the molecules (Table 3). Whereas the unsubstituted core structure **12a** was as active (K_i: 28 nM) as the comparable phenyl derivative **8b**, small or larger substituents R1 led to compounds weaker by a factor of 10 or more. Compounds **12d** and **12e**, both with substituents R1 and R2, had similar acitivity as the cyclopropyl derivative **12b**. In conclusion, substitution on the aryl ring seems to be detrimental for CCR3 receptor affinity.

Further variations of aryl ring substituents led to compounds **13**–**15** (Table 4). Whereas the furane **13** retained activity (compared to **12d**), the pyridine **14** and the imidazole **15** were considerably less active than the comparable substances **8b** and **12a**. Compounds with electron rich aryl rings seem to be more active than those with basic or electron poor ones. In summary, the SAR of the discussed structures is very narrow. Although the space to introduce further substituents is very limited, it is possible that polar substituents are beneficial for activity.

Compound **8b** stimulates freshly isolated human peripheral blood eosinophils via the CCR3 receptor as measured by the transient increase the of intracellular calcium concentration[19] (Fig. 1b). The same increase is seen with the natural agonist eotaxin-1 (Fig. 1a).

Furthermore, the CCR3 selective antagonist[8] RO320-2947/001 had no effect on intracellular calcium concentration. However, it completely inhibited eosinophil stimulation of compound **8b** (Fig. 1b) as well as that of eotaxin-1 (Fig. 1a).

Other compounds of this series (the imines **8a**,**c**,**f** and **12a**) showed essentially the same stimulation pattern depending on sufficient affinity to the CCR3 receptor (data not shown).

These findings clearly prove the agonist property of **8b** and strongly support that compound **8b** interacts via CCR3, that is, the same receptor as that used by eotaxin-1.

Acknowledgements

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18. The CCR3 receptor binding test is based on a K562 cell line (leukemia myelogenic blast cells) transfected with the human chemokine receptor CCR3 (hCCR3-C1). The cell membranes were prepared by disrupting the hCCR3 transfected K562 cells by nitrogen decomposition and centrifugation at 40,000*g*, 4 °C for 1 h. The membranes were resuspended in the SPA incubation buffer (see below) without bovine serum albumin for storage in aliquots at −80 °C.
The CCR3 receptor binding assay with the radioligand ^{125}Jodine-eotaxin-1 was performed in a Scintillation Proximity Assay (SPA) design. Cell membranes of hCCR3 C1 cells were diluted in suitable concentrations (0.5–5 μg protein/well) in 96-well microtiter plates (1450–401, Wallac).
The test incubation mixture comprising 60 μL of the membrane suspension, 80 μL of the Wheat Germ Agglutinin coated PVT beads (organic scintillator, Amersham Pharmacia biotech) in a concentration of 0.4 mg and 40 μL of radiolabelled ^{125}J rhEotaxin (Amersham, IM290) were incubated with 20 μL of the test compound (in DMSO dilutions) for 2 h. The SPA incubation buffer contained 25 mM HEPES, 25 mM $MgCl_2$ $6 \times H_2O$, 1 mM $CaCl_2$ $2 \times H_2O$ and 0.1% bovine serum albumin. Included were controls for specific binding (no displacer added) and nonspecific binding by adding unlabelled rhEotaxin (R&D Systems) or a test compound. Bound radioactivity was determined by scintillation counter ('Micro Beta Trilux', Wallac).
Determination of affinity of test compounds (dissociation constant K_i) was calculated by iterative fitting of experimental data using the law of mass action based program 'easy sys' Schittkowski *Num. Math.* **1994**, *68*, 129–142.

19. Eosinophil calcium influx test. *Eosinophil isolation*: The eosinophilic granulocytes were isolated from blood of healthy donors at 4 °C without Ca and Mg. ACD blood (containing citric acid, pH 5) was applied onto a Ficoll Paque (Pharmacia) layer. After centrifugation (300*g*, 25 min) the plasma and the Ficoll layer were removed and the PMNL/erythrocyte fraction lysed in Ammonium chloride solution. Eosinophils were isolated by negative selection in the MACS Separation System (Milteny) using magnetic beads conjugated to anti-CD16 antibodies. *Fura-2 cell loading*: Isolated eosinophils were incubated with the Ca^{2+}-indicator Fura-2 AM (2 μmol/L, Molecular Probes) for 45 min at 37 °C. After incubation, the cells were centrifuged at 300*g* for 3 min. Intracellular FURA-AM was hydrolyzed in a 15 min incubation at room temperature. *Measurement of cytoplasmic calcium concentrations:* The measurements were carried out with a fluorometer with an integrated pipettor module (Flexstation, Molecular Device) at the excitation wave lengths of 340 and 380 nm and an emission wave length of 510 nm. The ratio (340/380) is a measure of intracellular calcium concentration. The intensities of fluorescence (*F*) at the emission wave length 510 nm of Ca^{2+}-loaded Fura-2 after excitation at the wave lengths of 340 and 380 nm were measured and converted into the ratios *R* of Fura-2 fluorescence ($R = F_{340}/F_{380}$). These ratios are proportional to the intracellular calcium concentrations $[Ca^{2+}]_i$ and can be transferred into absolute calcium concentrations, Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. *J. Biol. Chem.* **1985**, *260*, 3440–3450.