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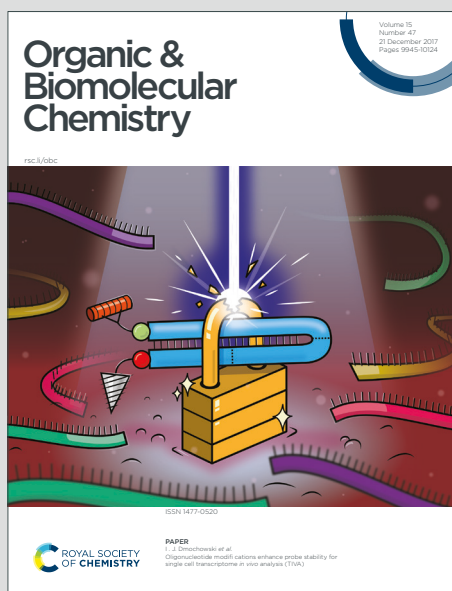
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# Organic & Biomolecular Chemistry

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## ARTICLE

## Selective synthesis of an elusive C-functionalized bis-cyclam and study of its inhibition properties of CXCR4 chemokine receptor

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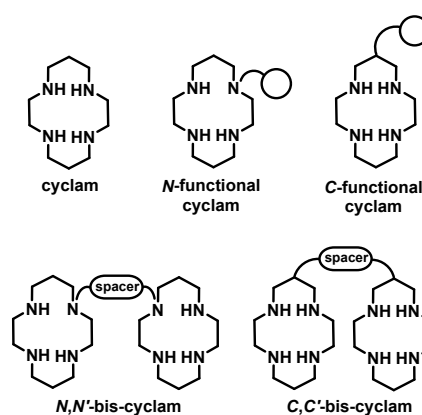
This article presents the controlled synthesis of a rare example of C,C'-linked bis-cyclam architecture in mild conditions through to the "bis-aminal" route, previously used for the advantageous synthesis of cyclam, N- and C-functional cyclams and N,N'-bis-cyclams. Two synthetic pathways were explored with the smart design of  $\alpha,\beta$ -unsaturated ketones or alkyl halides bis-cyclizing agents. The former lead to the isolation of a key intermediate for future design of N-functionalized bis-cyclams, whereas the latter allowed the preparation of the targeted C,C'-xylylene-bis-cyclam in mild conditions with decent yield. This compound was then studied as a CXCR4 receptor inhibitor, one of the main applications known for bis-macrocyclic compounds. Although results demonstrated that its potency is lower (i.e. 137-fold higher IC<sub>50</sub>) than golden standard AMD3100, clear evidence of CXCR4 inhibition is presented, confirming the potential of this novel architecture and related compounds in this research field.

### Introduction

Saturated polyazamacrocycles, such as **cyclen** (1,4,7,10-Tetraazacyclododecane) and **cyclam** (1,4,8,11 Tetraazacyclotetradecane), that are respectively 12- and 14-membered tetraamine rings, continue to be very attractive platforms, mainly due to their coordination properties towards a large range of cations.<sup>1</sup> For instance in the medical domain, they are the cornerstone for the development of novel medical imaging and therapeutic agents,<sup>2,3</sup> and they also demonstrated interesting properties for sensing or catalysis purposes.<sup>4,5</sup> In particular, among the starting platforms used to design stable chelators, **cyclam** proved to be the best suited azamacrocycle for transition metal cations such as copper(II), nickel(II), zinc(II) or cobalt (II), as it offers the best match between geometry and size with these divalent metallic cations thanks to a perfect combination of macrocyclic and chelate effects.<sup>6,7</sup> Depending on the application, additional functional groups can be introduced through N- or C- functionalization (Scheme 1), to add supplementary coordinating groups and/or allow grafting on an external support<sup>8,9</sup> or biomolecules for medical use.<sup>10,11</sup> Their ditopic analogues, the bis-cyclams, have also attracted interest in the last decades. Some were used for their coordination properties for transition metals and anions,<sup>12–17</sup> but also for their spectacular anti-Human Immunodeficiency Virus (anti-HIV) activity, that was first

discovered in the 1990s.<sup>18,19</sup> In this case, their potency has been attributed by some of us, among others, to their inhibition properties of the CXCR4 chemokine receptor,<sup>20–22</sup> a key cofactor in the HIV virus cell-entry process.<sup>23</sup>

Although such macrocyclic compounds have low molecular weight and small number of atoms, their synthesis is rather challenging, as selective ring closing reactions are required. Indeed, if **cyclam** and other azamacrocycles were first isolated in the early 20<sup>th</sup> century in low yields through statistical ring-forming reactions,<sup>24</sup> the first high-yielding syntheses of 9- to 21-membered polyazamacrocycles were only described in the 1970s by Richman and Atkins,<sup>25,26</sup> inspired by previous work from Stetter *et al.*<sup>27,28</sup> This



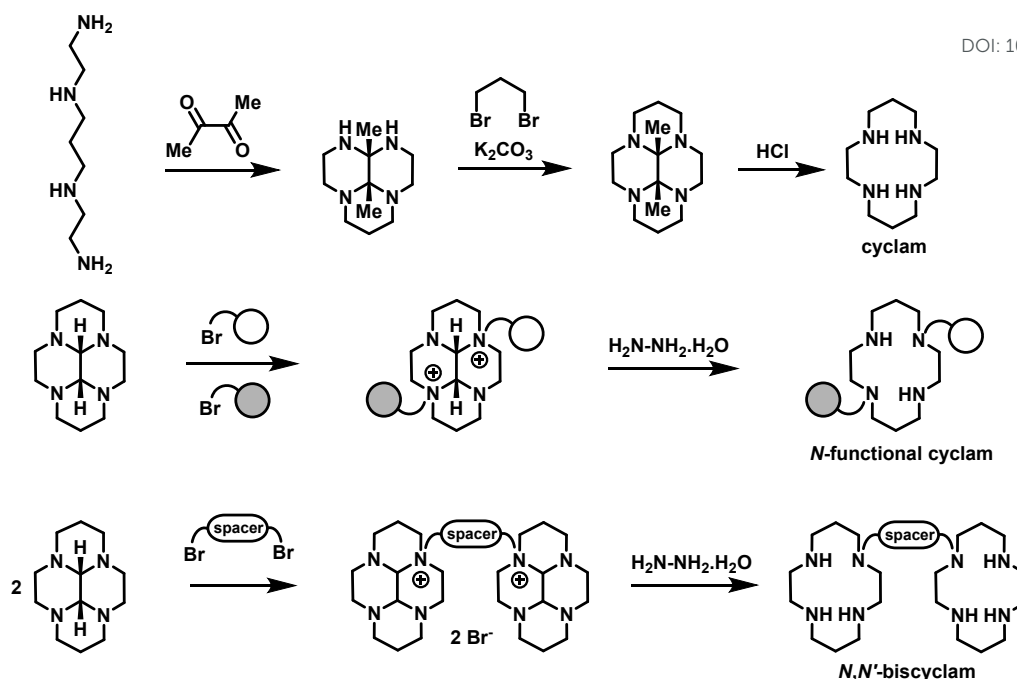
**Scheme 1.** Schematic structure of **cyclam**, N/C-functional and ditopic analogues.

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Electronic Supplementary Information (ESI) available: Synthetic protocols, High-resolution Mass Spectra, <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy of all new compounds, X-ray diffraction analysis when available. See DOI: 10.1039/x0xx00000x



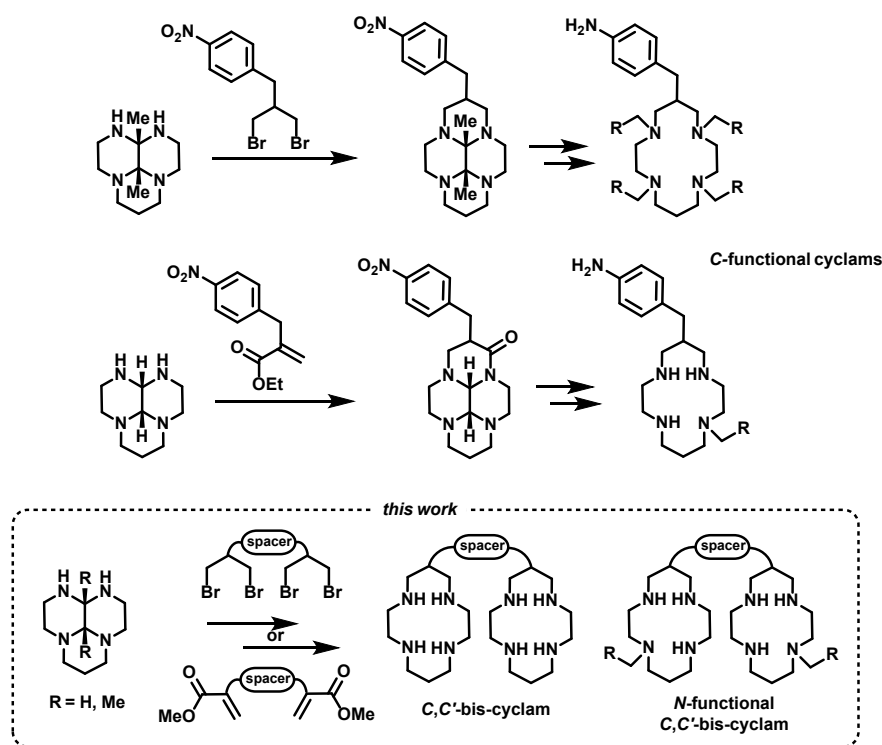
**Scheme 2.** Synthesis of **cyclam**, its *N*-functional and *N,N'*-ditopic analogues via the “bis-aminal” routes.

method relies on the derivatization of open-chain polyamines as rigid sulphonamides bearing tosyl groups, followed by a ring-closing reaction. Mono-*N*-functionalization of cyclam was developed following the same strategy, with the use of protecting groups such as tosyl, tert-butoxycarbonyl (Boc) or trifluoroacetyl moieties, and so were the first syntheses of *N,N'*-bis-cyclams.<sup>29</sup> Although these reactions are efficient, the atom-economy and reaction conditions (high temperatures, long reaction time, use of concentrated acids) are not optimal. Metal-assisted templation of linear polyamines with nickel(II) cations was also explored in order to soften reaction conditions,<sup>30,31</sup> but this strategy has the drawback of using reactive and toxic species (nickel and cyanide salts, Raney nickel). Finally in 1998, the “bis-aminal” route was described, using a two-carbon atoms organic template, that first allowed preparation of **cyclam** in two steps with 90 % yield, with fast reactions in mild conditions (Scheme 2, top).<sup>32</sup> Interestingly, the same synthetic tool allowed regiospecific mono- and di-*N*-functionalization of cyclam, as well as the preparation of *N,N'*-bis-cyclams with various spacers, thanks to the selective formation and isolation of ammonium intermediates allowed by the bis-aminal structure (Scheme 2, middle and bottom), providing dimerization yields greater than 80 %.<sup>33,34</sup>

Recently some of us, among others, have also profited from such bis-aminal templates to synthesize elusive *C*-functional cyclam analogues, through ring-closing reactions with bis-halides or  $\alpha,\beta$ -unsaturated ketones (Scheme 3).<sup>35–37</sup> These strategies permitted in particular concomitant *N*- and *C*-functionalizations leading to the selective preparation of tetra- and mono-*N*-functionalized ligands respectively, with coordination properties of the nitrogen atoms being unaltered by the *C*-functionalization, that allows conjugation to biomolecules. These highly valuable bifunctional chelators have been further used in the design of innovative radiopharmaceuticals

for targeted imaging and therapy in nuclear medicine.<sup>38–40</sup> However, within this wide range of cyclam and bis-cyclam derivatives, *C,C'*-bis-cyclam compounds remain very scarce and, to the best of our knowledge, only two *C*-functional bis-cyclam structures have been prepared in the literature so far: a direct *C-C'* linked structure (no spacer) first prepared by Fabbrizzi *et al.*,<sup>41,42</sup> and a tetramethylated *o*-xylylene architecture prepared by Ito *et al.*<sup>43,44</sup> through the nickel-templated strategy, leading to difficult removal of the cation and therefore mainly studied as the di-nickel(II) complex.<sup>45–47</sup> Thus, we sought to explore the synthesis of such architectures through the bis-aminal routes in mild conditions, thanks to the design of bifunctional cyclizing  $\alpha,\beta$ -unsaturated ketones or alkyl halides, inspired by previous *C*-functional cyclam syntheses (Scheme 3, bottom). The latter is expected to allow the synthesis of naked *C,C'*-bis-cyclams, where the former could also selectively provide analogues with mono-*N*-functionalized cyclam units to further enlarge the scope of this novel class of bis-macrocyclic compounds.

When it comes to applications of bis-cyclams, their anti-HIV properties are quite appealing. Historically, the first bis-macrocyclic identified as a potent anti-HIV drug has been *C-C'*-bis-cyclam **JM1657** (Scheme 4), that was serendipitously identified as an impurity in commercial **cyclam** batches.<sup>48</sup> Its half maximal inhibitory concentration ( $IC_{50}$ ), *i.e.* the concentration required to inhibit 50 % of HIV infection, was determined at 0.144  $\mu$ M. However, this compound could not be specifically prepared owing to the difficult synthesis of *C,C'*-bis-cyclams, and *N,N'*-derivatives were thus considered instead. Structure-Activity Relationship (SAR) successively identified *N,N'*-propylene-bis-cyclam **JM2763** as an alternative ( $IC_{50}$  = 0.248  $\mu$ M), but *N,N'*-*m*-xylylene bis-cyclam (**AMD3100**, commercialized as Plerixafor<sup>®</sup>) was finally found to be the most active ( $IC_{50}$  = 0.003  $\mu$ M) compound of the family so far.<sup>19,29,49,50</sup> In addition to their anti-HIV



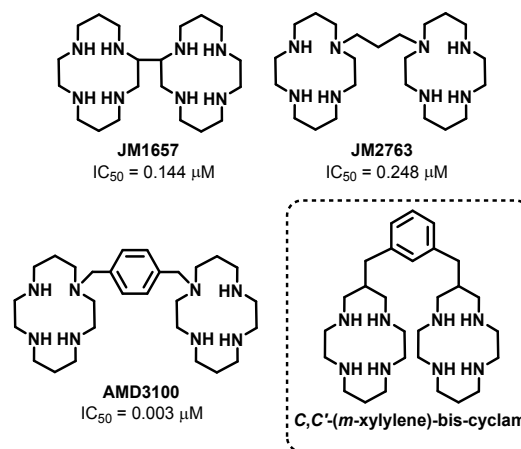
**Scheme 3.** Synthesis of *C*-functional cyclam chelators through bis-aminal route, and *C,C'*-bis-cyclam synthetic strategy

activity, the high clinical potential of these CXCR4 inhibitors has also been studied in the context of other pathologies, and cancer in particular, due to the overexpression of the CXCR4 receptor in more than 20 types of human tumours.<sup>51</sup> For instance, functional groups have been added on the **AMD3100** platform (on aryl spacer of secondary amines) to alter its cellular interactions and biodistribution,<sup>52–56</sup> or develop potential CXCR4-targeted imaging agents,<sup>57–60</sup> further demonstrating the potential of such small organic compounds in modern medicine. The molecular interaction of **AMD3100** with CXCR4 receptor has been extensively studied, demonstrating that flexibility of the compound and hydrogen bonding involving the protonated secondary amines of the cyclam moieties are crucial in the inhibition of the receptor.<sup>61–63</sup> As aforementioned, a large range of **AMD3100** analogues has been synthesized, but structural variations have been focused on the spacer (length, nature) and the macrocycle size, but the spacer location has been restricted to *N,N'*-functionalizations of the cyclam units. In addition, derivatization of secondary amines to tertiary alkylamines on the cyclam units proved detrimental for the recognition properties.<sup>29,54</sup> Therefore, we envisioned that a bis-cyclam with a *C,C'*-xylylene link could strongly interact with the receptor thanks to its unique combination of eight secondary amines (as in **JM1657**, Scheme 1), and suitable flexibility provided by the spacer (as in **AMD3100**). *m*-xylylene spacer was selected on the basis of simple molecular modelling, as the *p*-xylylene (as in **AMD3100**) is expected to move the two cyclam moieties too far away compared to **AMD3100**, whereas the *o*-xylylene one would provide strong steric hindrance between the two macrocycles resulting in a loss of overall flexibility. Thus, we present herein the synthesis of the first *C,C'*-(*m*-

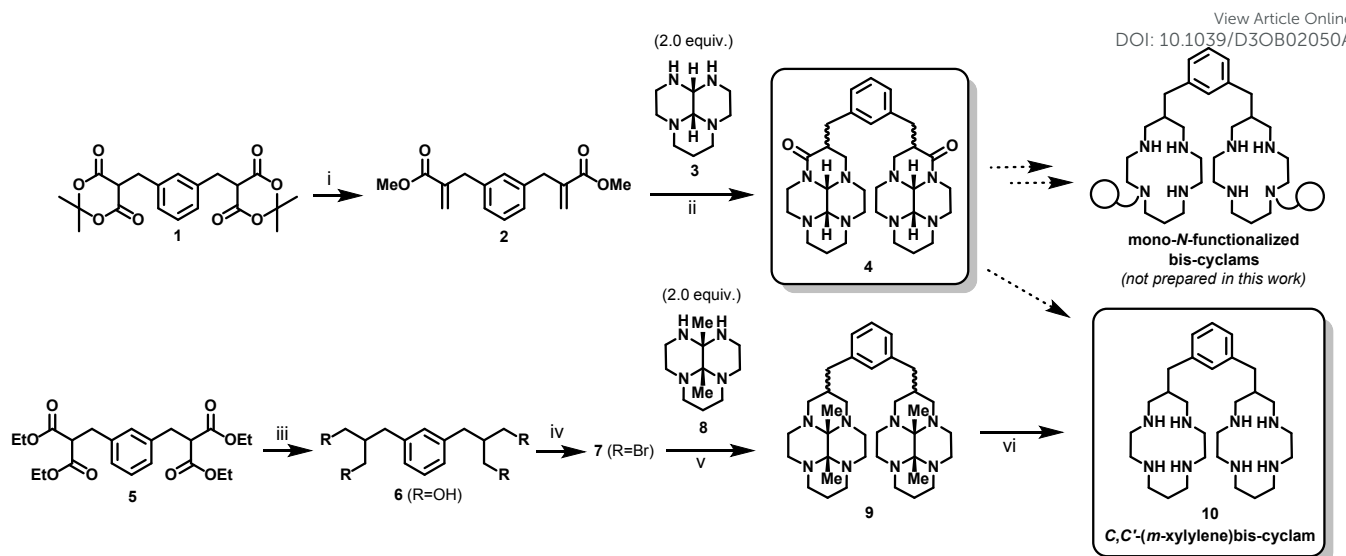
**xylylene)-bis-cyclam** (Scheme 4, right), and subsequent CXCR4 recognition experiments as a proof-of-concept for the potential of such *C,C'*-bis-cyclams in the domain. A key intermediate for the future synthesis of *N*-functional analogues is also described.

## Results and Discussion

The first synthetic pathway using  $\alpha,\beta$ -unsaturated ketones with bis-aminal intermediate **3** was adapted from our previous work on the synthesis of *C*-functional cyclam derivatives,<sup>36,40</sup> starting from compound **1** that could be prepared according to literature<sup>64</sup> through reductive Knoevenagel condensation of Meldrum's acid and



**Scheme 4.** Structure of bis-cyclams **JM1657**, **JM2763**, **AMD3100**, and *C,C'*-**xylylene-bis-cyclam** (presented in this work). The presented  $IC_{50}$  values are their inhibition values in  $\mu M$  on CXCL12-induced  $Ca^{2+}$ -signalling (*vide infra*).



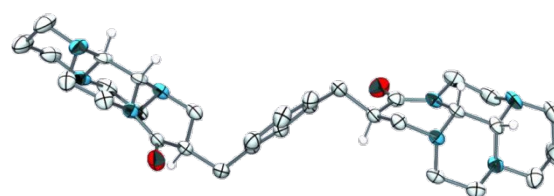
**Scheme 5.** Synthetic routes to **10** (*C,C'*-(*m*-xylylene)bis-cyclam) via cyclization with  $\alpha,\beta$  unsaturated ketones or bis(dihalides). Reaction conditions and yields: i) Eschenmoser's salt (5.0 equiv.), MeOH, 65 °C, 12 h (97 %); ii) EtOAc, RT, 4 months (30 %); iii) LiAlH<sub>4</sub> (6.0 equiv.), -10 °C to RT then 50 °C for 18 h (91 %); iv) CBr<sub>4</sub> (20 equiv.), PPh<sub>3</sub> (20 equiv.), DMF, RT (63 %); v) K<sub>2</sub>CO<sub>3</sub> (20 equiv.) CH<sub>3</sub>CN, 60 °C, 10 days (16 %); vi) HCl (3M), RT, 24 h (59 %).

isophthalaldehyde in presence of Hantzsch ester (Scheme 5). This product was further reacted with Eschenmoser's salt leading to bis( $\alpha,\beta$ -unsaturated)-diketone **2** in almost quantitative yield. These two intermediates were fully characterized by means of <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy as well as high-resolution mass spectroscopy (HRMS) (Figures S1-S4, Supporting Information). This cyclizing agent was reacted with templated **3**, mirroring the reaction described for the synthesis of *C*-functional cyclam. Where the latter already requires long reaction time (> 14 days at RT),<sup>36</sup> in our case the double cyclisation leading to **4** occurred only with modest yield (30 %) after 120 days at room temperature in ethyl acetate. Other solvents suitable for this reaction (tetrahydrofuran, acetonitrile) were investigated, but did not lead to any conversion. Noteworthy, temperature had to be kept at 25 °C, as the 2-carbon atoms bridge in **3** can easily isomerize from *cis* to *trans* geometry when heated, with the generated *trans* arrangement being too stable to be deprotected after cyclization.<sup>65</sup> Nevertheless **4** was isolated and fully characterized by NMR spectroscopy, with <sup>1</sup>H and <sup>13</sup>C evidencing the diastereoselectivity of the reaction with only one set of signals observed, and diagnostic <sup>13</sup>C NMR spectroscopy singlets at 76.0, 70.6 and 37.0 ppm corresponding to both aminal -CH- and chiral  $\alpha$ -amido -CH- groups respectively (Figure S5, Supporting Information).

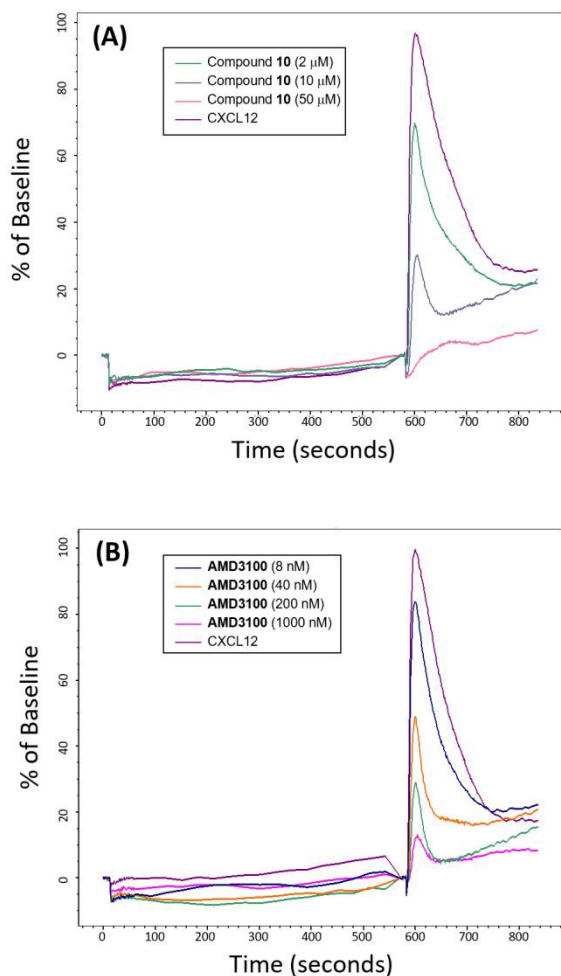
This sole product was identified as the *cis-syn/cis-syn* isomer (*i.e.* *cis*-aminal bridge and xylylene moiety pointing towards the same face of the macrocyclic plane for both cyclam units) thanks to X-Ray diffraction analysis on single crystals grown from an ethyl acetate solution. NMR spectroscopy was also performed on the crystals used for X-Ray diffraction, confirming that this solid state structure reflected the whole isolated batch of compound **4**. This new cyclized product could then lead to targeted *C,C'*-(*m*-xylylene)bis-cyclam through four extra steps, already described for mono-cyclam analogues,<sup>36</sup> but is especially a crucial intermediate for the

preparation of *N*-functional analogues. Indeed, the presence of the carbonyl group allows to promote regioselective mono-*N*-functionalization, as previously described for *C*-functional cyclams,<sup>36</sup> potentially leading to a large scope of interesting *C,C'*-bis-cyclams. However, due to the long reaction required to obtain this product, further optimization is required, and our efforts in this work were then focussed on the second pathway using dimethylated bis-aminal **8** and corresponding dihalide cyclizing moieties (Scheme 5). Dimalonyl-*m*-xylylene **5** was prepared via a nucleophilic substitution reaction between  $\alpha,\alpha'$ -dibromo-*m*-xylylene and diethyl malonate in presence of sodium hydride, and was further reduced to tetraalcohol **6** by reaction with lithium aluminium hydride (Scheme 5, bottom). Alcohol functions were then derivatized to better-leaving bromide groups by an Appel reaction with carbon tetrabromide in presence of triphenylphosphine as a bromide-transfer agent.

Tetrabrominated derivative **7** was thus obtained on 500 milligram scale in three days with good overall yield (31 % over three steps) from commercial reagents. New compounds **6** and **7** were fully characterized by HRMS and NMR spectroscopy, with -CH<sub>2</sub>OH (<sup>1</sup>H: 3.54 ppm, <sup>13</sup>C: 63.1 ppm) and -CH<sub>2</sub>Br (<sup>1</sup>H: 3.43 ppm, <sup>13</sup>C: 35.8 ppm)



**Figure 1.** Single crystal X-ray structure of **4** (ellipsoids are plotted at the 50 % probability level; H-atoms excepted those involved in chirality) and solvent molecules omitted for clarity.



**Figure 2.** Antagonistic effect of compound **10** and the reference compound **AMD3100** on calcium signalling in CXCR4-transfected human U87 glioblastoma cells. The inhibitory effect of several concentrations of compound **10** (Panel A) and **AMD3100** (Panel B) on CXCL12-induced calcium release is shown. Compound **10** or **AMD3100** were added at  $t = 0$  and CXCL12 (50 ng/mL) was added to each sample at  $t = 600$  s. The highest response represents the calcium release from untreated cells stimulated with CXCL12 (positive control). Calcium responses are represented as % of baseline (*i.e.* mean relative light units in each well). Each datapoint corresponds to the mean fluorescence of three replicates. One representative experiment out of three is shown.

signals allowing the easy monitoring of the Appel reaction progress and ensure selective formation of compound **10** (Figures S9-S11, Supporting Information). The double ring-closing reaction with two equivalents of bis-aminal templated tetramine **8** was then carried out, with heating at 60 °C allowed in this case as the methylated bis-aminal bridge prevents the *cis/trans* isomerization process observed in the case of bis-aminal **3** (*vide supra*). After 10 days, product **9** was isolated from the reaction mixture by column chromatography on silica gel and obtained as a white solid with a modest 16 % yield (84 mg). In this case, the reaction is not diastereoselective and a mixture of isomers was observed. However, after hydrolysis in acidic medium (3M HCl), **10** was obtained (59 % yield) as one pure product as no chiral center remains on its structure once the bis-aminal bridge is cleaved. This compound was fully characterized by HRMS and NMR spectroscopy with the functionalized  $-CH-$  group from the cyclam scaffold and the methylenic  $-CH_2Ar$  each exhibiting one singlet resonance ( $^{13}C$ : 35.8 and 38.4 ppm respectively), confirming the symmetry of the molecule. Unfortunately, despite several attempts, no single crystals of this compound could be grown to confirm its structure by X-Ray diffraction analysis. Nevertheless, **10** was obtained on 70 milligram scale, allowing to tackle the first CXCR4 inhibition cellular assays.

The activity of **10** as a CXCR4 inhibitor was evaluated in different assays by comparison with the receptor's natural ligand CXCL12. One of the main signalling pathways upon stimulation of CXCR4 by CXCL12 is an intracellular calcium release. A first screen of compound **10** was therefore carried out using a calcium signaling assay to determine the capacity of compound **10** to block intracellular calcium release in response to stimulation. This experiment allows to evaluate both the potential agonistic (in absence of CXCL12) as well as the antagonistic activity (*i.e.* the potency to inhibit the CXCL12-induced calcium mobilization) of compounds in real time using the FLIPR Tetra System (Molecular Devices®) according to previously described methodology.<sup>66</sup> The inhibition of the calcium flux was measured at a series of concentrations to determine the value whereby a 50 % reduction in calcium response was observed (Table S2, Supporting Information). **AMD3100** was used as reference compound. Compound **10** showed micromolar antagonistic activity ( $IC_{50} = 3.58 \mu M$ ) but was clearly less potent than **AMD3100** ( $IC_{50} = 0.14 \mu M$ ). No agonistic activity on CXCR4 for both compounds was observed at a concentration up to 100  $\mu M$ . The ability of compound **10** to inhibit the CCR5 receptor (the second co-receptor involved in HIV-infection) was also explored, as some CXCR4 inhibitors have also proven potent for this receptor in the past.<sup>67</sup> Monitoring of the calcium response in U87.CCR5+ cells induced by stimulation with a potent CCR5-specific chemokine (LD78beta) did not reveal any inhibitory activity ( $IC_{50} > 20 \mu M$ , Figure S17, Supporting Information), similarly to what is observed for **AMD3100**.

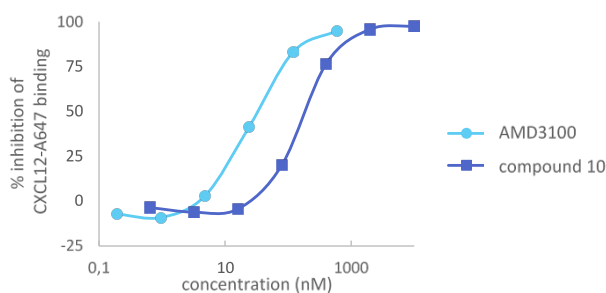
The concentration-dependent inhibition of the CXCL12-induced calcium response showed that compound **10** clearly inhibits the binding of its natural ligand to the CXCR4 receptor. These findings were confirmed by a CXCL12 binding assay. Here, human Jurkat cells endogenously expressing CXCR4, were pretreated with serial dilutions of compound **10** and thereafter incubated with fluorescent-

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labeled human CXCL12<sup>AF647</sup>. Here again **AMD3100** was used as a reference. The fluorescent signal of the CXCL12<sup>AF647</sup> binding was analysed by flow cytometry as described in detail in the Experimental Part (*vide infra*). Both **10** and **AMD3100** inhibited the binding of CXCL12<sup>AF647</sup> in a dose-dependent manner (see Figure 3) with IC<sub>50</sub> values in the nanomolar range, namely 194 nM for compound **10** and 34 nM for **AMD3100** (Figure S18, Supporting Information).

Subsequently, to gain somewhat more insight into the critical binding sites of compound **10** on the CXCR4 receptor a binding assay using monoclonal antibodies (mAbs) directed against different regions (epitopes) of the CXCR4 receptor was performed (Figure S19 and Table S3, Supporting Information). The 12G5 mAb mainly targets the second extracellular loop while the 2B11 mAb binds to the amino-terminal loop of the CXCR4 receptor.<sup>61,68–70</sup> For this set of experiments, human Jurkat cells endogenously expressing CXCR4 were used and pretreated with serial dilutions of compound **10** and reference compound **AMD3100** and thereafter incubated with the phycoerythrin-labeled 12G5 and 2B11 mAbs. The strength of the fluorescent signal of the CXCR4 mAb binding was analysed by flow cytometry. Compound **10** blocked the 12G5 mAb binding to CXCR4 (IC<sub>50</sub> = 376 nM) and was not able to inhibit the 2B11 mAb binding (IC<sub>50</sub> > 20 μM). These results suggest that compound **10** interacts with comparable binding sites on CXCR4 as **AMD3100**, as this compound potently inhibits 12G5 mAb binding (IC<sub>50</sub>: 6.8 nM) but not at all 2B11 mAb binding (IC<sub>50</sub> > 20 μM).



**Figure 3.** Dose dependent inhibition of CXCL12<sup>AF647</sup> binding by compound **10** and **AMD3100** evaluated in Jurkat.CXCR4+ cells

As mentioned before, CXCR4 also plays an important role in human immunodeficiency virus (HIV) entry where it functions as a crucial co-receptor, alongside CC chemokine receptor 5 (CCR5), for HIV infection and replication in CD4+ T cells.<sup>23</sup> To investigate the possible anti-HIV-activity of compound **10**, we performed HIV replication assays in specific-transfected TZM-bl cells. These cells stably express both CD4 receptor as well as the HIV co-receptors CXCR4 and CCR5. Inhibition of HIV infection can be measured as a reduction in HIV-1 Tat-regulated firefly luciferase reporter gene expression.<sup>71</sup> Briefly, TZM-bl cells were pre-incubated with varying concentrations of compound **10** and reference compound **AMD3100** for 30 min before HIV-1 NL4.3, a CXCR4 co-receptor using HIV-1 strain (X4), or HIV-1 BaL, a CCR5 co-receptor using HIV-1 strain (R5), were added. After an additional 48 h incubation period, luminescence was measured after

10 min of incubation with SteadyLite Plus reagent (PerkinElmer) and values from the sample-treated wells were compared with those of the untreated virus-infected cells. IC<sub>50</sub> were calculated, being the concentration of the compound that reduces the relative luminescence units by 50 % compared with the virus control. In parallel, cellular cytotoxicity of the compound was also determined following the same protocol but without adding virus to the cells. These sets of experiments showed that compound **10** could successfully prevent infection of TZM-cells by the HIV-1 NL4.3 (X4) strain with an IC<sub>50</sub> value of 96 nM. The reference compound **AMD3100** blocks (as described previously) the HIV-infection very potently with an IC<sub>50</sub> value of 0.7 nM. Both compounds showed no cellular cytotoxic effects at the highest concentration tested (100 μM). As expected, both compound **10** and **AMD3100** had no activity (> 20 μM) against HIV-1 BaL replication which indicates again that they do not interact with the CCR5 receptor and confirms their observed inactivity in the CCR5-induced calcium signalling experiments.

## Conclusions

Building on our experience in the preparation of C-functional cyclam derivatives, we developed new synthetic pathways to access elusive C,C'-bis-cyclams, in order to assess their potential as CXCR4 inhibitors. Thanks to the bis-aminal synthetic route, we could isolate a first example of such architecture, C,C'-(m-xylylene)-bis-cyclam, and carried out the first biological assays for CXCR4 receptor recognition for this new family of compounds. This bis-cyclam could be prepared in mild conditions on a 70 mg scale thanks to alkyl halide double cyclizing agents. Biological assays were then conducted with **AMD3100**, that has been the most potent organic CXCR4 inhibitor for over two decades, as a reference. To assess the inhibition potential of the new compound, calcium mobilization, direct CXCL12 inhibition and HIV-replication assays have been conducted, all confirming the inhibition properties of C,C'-(m-xylylene)-bis-cyclam, with receptor interactions mirroring the one of **AMD3100** at the molecular scale. The magnitude of this inhibition is somewhat lower than the one of **AMD3100** (between one and two orders of magnitude lower in the different assays), but confirms that this new family of bis-cyclam compounds can be used in that context, and paves the way for further structural modifications. For that objective, the second synthetic route allowed us to isolate the C,C'-(m-xylylene)-bisoxocyclam intermediate through the use of a double α,β-unsaturated ketone as cyclizing agent, and could be the starting point for the development of selectively N-functionalized-C,C'-bis-cyclams and subsequent SAR studies of such compounds towards CXCR4 receptor inhibition.

## Experimental Section

**X-ray Diffraction.** Crystallographic data were collected at the Service RX of the Institut de Chimie de Toulouse, Université Paul Sabatier, on a Bruker-AXS kappa APEX II Quazar diffractometer using MoKα radiation (λ = 0.71073 Å) at 193(2) K. Space groups were determined

	Toxicity CC <sub>50</sub> (μM)	HIV-1 NL4.3 (X4) IC <sub>50</sub> (μM)	HIV-1 BaL (R5) IC <sub>50</sub> (μM)
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<b>Compound 10</b>	>100 ± 0	0.096 ± 0.020	>20 ± 0
<b>AMD3100</b>	>100 ± 0	0.0007 ± 0.00009	>20 ± 0

**Table 1.** Antiviral activity of compound **10** and **AMD3100** against the HIV-1 strains NL4.3 (X4) and HIV-1 BaL(R5) in TZM-bl luciferase reporter gene infection assay.



based on systematic absences and intensity statistics. Semi-empirical absorption correction was employed. The structure was solved using an intrinsic phasing method (SHELXT)<sup>72</sup> and refined using the least-squares method on F<sub>2</sub>.<sup>73</sup> All non-H atoms were refined with anisotropic displacement parameters. Hydrogen atoms were refined isotropically at calculated positions using a riding model with their isotropic displacement parameters constrained to be equal to 1.5 times the equivalent isotropic displacement parameters of their pivot atoms for terminal sp<sup>3</sup> carbon and 1.2 times for all other carbon atoms. CCDC-2225027 (compound **4**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via <https://www.ccdc.cam.ac.uk/structures/>.

**Calcium signaling receptor assay.** Intracellular calcium responses were measured with the fluorescent and luminescent imaging plate reader (FLIPR) Penta system (Molecular Devices). Human U87.CD4.CXCR4 cells were cultured in DMEM 10 % FBS and seeded in 0.1 % gelatin-coated 96-well black-wall transparent-bottom microplates at 20000 cells per well (200  $\mu$ l). The next day, culture medium was removed and plated monolayers were loaded with the fluorescent calcium indicator Fluo-2 in assay buffer) for 45 min at room temperature in the dark. During this incubation period, a 96-well plate with varying concentration of compounds, diluted in assay buffer (HBSS, Hanks' balanced salt solution, Thermo Fisher Scientific), 40 mM HEPES buffer (Thermo Fisher Scientific), 0.5 % FCS (Thermo Fisher Scientific), pH 7.4), and a 96-well plate with the ligand CXCL12 (50 ng/ml) were prepared. After washing with assay buffer, the cells were preincubated for 1 min at 37 °C in the FLIPR Penta System. At the start of the measurement, compounds were added to the cell plate by the FLIPR system followed by an incubation period of 10 min during which the intracellular calcium level was continuously measured. Following this incubation, CXCL12 was added and changes in cytosolic calcium concentration of the cells were simultaneously measured by the FLIPR system.

**CXCL12<sup>AF649</sup> receptor binding assay.** 2x10<sup>5</sup> human Jurkat cells, resuspended in assay buffer (Hanks' Balanced Salt Solution HBSS, 20 mM HEPES, 0.5 % FBS, pH 7.4) were treated with different concentrations of compounds in a clear 96-well round bottom plate for 15 min at RT. Next, 50  $\mu$ l of 100 ng/ml Alexa Fluor-649 labeled CXCL12 (Almac), diluted in assay buffer, was added and the cells were incubated at RT for 30 min in the dark. After washing in assay buffer the cells were fixed in 1 % paraformaldehyde in DPBS. Specific CXCL12<sup>AF647</sup> binding was quantified by flow cytometry (FACSCanto™; Becton Dickinson). Data were analyzed with FlowJo™ Software. The 50 % inhibitory concentration (IC<sub>50</sub>) was calculated relative to the negative (i.e. untreated and unlabeled cells) and positive (i.e. cells treated with CXCL12<sup>AF549</sup> only).

**Mabs (12G5 and 2B11) binding inhibition towards CXCR4.** 1x10<sup>5</sup> Jurkat cells, resuspended in assay buffer (Hanks' Balanced Salt Solution HBSS, 20 mM HEPES, 0.5 % FBS, pH 7.4) were treated with different concentrations of compounds in a clear 96-well round bottom plate for 15 min at RT. Next, anti-CXCR4-PE clones 12G5 (Becton Dickinson) and 2B11 (Becton Dickinson) were added and cells were incubated at RT for 30 min in the dark. After washing in

assay buffer solutions the cells were fixed in 1 % paraformaldehyde in DPBS. Specific CXCR4 mAb binding was quantified by flow cytometry (FACSCanto™; Becton Dickinson). Data were analyzed with FlowJo™ Software. The 50 % inhibitory concentration (IC<sub>50</sub>) was calculated relative to the negative (i.e. untreated and unlabeled cells) and positive (i.e. CXCR4+ cells stained with 12G5 or 2B11 mAb only).

**HIV-1 replication assay.** 1.7 x 10<sup>4</sup> TZM-bl cells were pre-incubated with serial concentrations of compounds in DMEM cell culture medium supplemented with diethylaminoethyl-dextran (15  $\mu$ g/ml; Sigma-Aldrich) in 96-well-plates at 37 °C for 30 min in a final volume of 200  $\mu$ l. Then the cells were infected with HIV-1 NL4.3 (X4) and HIV-1 BaL (R5) titrated virus stocks. After 48 h of incubation, 120  $\mu$ l of supernatants was removed from each well and 65  $\mu$ l of Steady-lite Plus Substrate solution (Perkin Elmer, Waltham, MA, USA) was added to the wells. Plates were shaken for 10 min (in the dark) before the luminescent signal, which is proportional to the level of viral replication, was measured using the Spectramax L microplate reader (Molecular Devices) and analyzed using Softmax Pro software (Molecular Devices).

## Author Contributions

TT, RT and DS conceptualized the project and supervised its realization. MLR and SC conducted investigations on the chemical synthesis/characterization and biological studies respectively. NSM conducted X-Ray diffraction analysis. TT wrote the original draft and all co-authors contributed to the reviewing & editing process.

## Conflicts of interest

There are no conflicts to declare.

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## Notes and references

‡ CCDC-2225027 contains the supplementary crystallographic data for compound **4**. (<https://www.ccdc.cam.ac.uk/structures/>).

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