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Synthesis of C-linked immobilized analogs of aloisine A by 'click' chemistry

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ABSTRACT

An efficient approach for the immobilization of a series of analogs of aloisine A, an in vitro inhibitor of protein kinases, to polymeric supports via a [3+2] cycloaddition reaction is reported.

Cyclin-dependent kinases (CDKs) belong to the large family of protein kinases that catalyze the phosphorylation of proteins at a Ser/Thr residue. CDKs are involved in numerous biological processes such as cellular differentiation, transcription, apoptosis, and cell cycle control.[1,2] Furthermore, CDKs have been linked to several diseases, for example, cancer, neurodegenerative diseases, and diabetes.[3] Due to their key roles, tremendous efforts have been engaged to identify potential inhibitors of CDKs.[4] The selectivity of kinase inhibitors is usually addressed by testing inhibitors on a panel of purified kinases.[5] However, even the most sophisticated panels cannot cover all kinases of the human genome, of which more than 800 have been postulated. Furthermore, other non-kinase targets need to be considered, either for detrimental or for synergistic effects.[6] Alternative approaches have therefore been developed to assess the selectivity of kinase inhibitors. Of these, affinity chromatography[7] of cellular extracts on immobilized inhibitors has previously been applied successfully to purvalanol,[8] paullone,[9] indirubin,[10] and roscovitine.[11]

Aloisine A (7-*n*-butyl-6-(4-hydroxyphenyl)-5*H*-pyrrolo [2,3-*b*]pyrazine, Fig. 1) is a potent inhibitor of CDKs, highly selective for CDK1/cyclinB, CDK2/cyclin A-E, CDK5/p25, and GSK-3 (Glycogen synthase kinase-3) based on in vitro assays.[12,13] A thorough investigation of the selectivity of aloisine is of interest for a better understanding of its cellular and physiological action, as well as the optimization of its pharmacological properties. However, to take into account the potential perturbations arising from introduction of a linker tail necessary for immobilization, various positions of the molecule have to be considered. In our previous work, we have synthesized aloisine conjugates bearing an extended tri(ethyleneglycol) (TEG) linker either on the butyl chain or in the 4′-hydroxyl position to assess its selectivity by affinity chromatography.[14] In this Letter, we report the synthesis of aloisine conjugates bearing a TEG chain in the C-3′ position. Furthermore, the synthetic strategy developed, based on the well-documented Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition (so-called 'click' reaction),[15,16] also allows for the direct immobilization of a series of aloisine analogs onto beads.

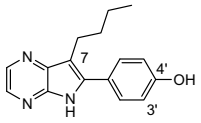

Figure 1. Aloisine A.

The synthesis of pyrrolo[2,3-*b*]pyrazine cores typically involves the condensation of a benzonitrile derivative with a lithiated alkyl pyrazine.[17] Introduction of the triethylene glycol linker can be performed either on the pyrrolo[2,3-*b*]pyrazine or, in most cases, on its benzonitrile or pyrazine precursor. However, numerous problems were observed using the combination of a basic and hygroscopic pyrazine core and a chelating, hydrophilic TEG linker,[14] which we sought to overcome by incorporating the chain at a very late stage. Our strategy thus relies on the preparation of the 3′-alkynyl aloisine **2** as key intermediate, allowing subsequent conjugation with the azido-functionalized TEG linker **3**[18] (Fig. 2).

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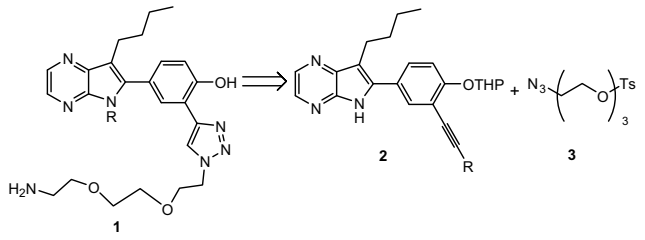


Figure 2. Strategy for the introduction of a TEG linker at *C*-3′ position.

The synthesis of **1** is depicted in Figure 3. The commercially available 3-bromo-4-hydroxybenzonitrile **4** was protected as the THP ether by reaction with DHP in the presence of PPTS in dichloromethane to afford **5** in 57% yield. **5** was then reacted under Sonogashira conditions with (trimethylsilyl)acetylene to give the 3-alkynylbenzonitrile **6** in 77% yield.[19] Compound **6** was reacted with pentylpyrazine in the presence of 2 equiv of LDA, to provide a mixture of the expected **7** and its desilylated product **2**.[20,21] Treatment of this mixture with TBAF allowed obtention of the acetylene **2** in 80% yield over two steps. The key cycloaddition step of **2** with the azido chain **3** was performed using CuI and DIPEA in acetonitrile at room temperature and afforded the triazole **8** in 85% yield.[22] Nucleophilic substitution of the tosyl with sodium azide gave **9** (77%), which upon cleavage of the 4′-OTHP with *p*-TSA, and reduction of the azide gave the amino-terminated linked aloisine **1** in 64% yield. This route proved to be considerably more efficient than those in which the TEG chain was incorporated at an early stage.

In view of the encouraging results obtained by this approach, we then explored the possibility of using the cycloaddition reaction directly on solid-phase to immobilize the molecule onto beads. Indeed, several examples have shown the potential of the copper-catalyzed 'click' reaction on solid support under a broad range of conditions.[23–25] Such an approach would only require incorporating a relatively inert alkyne functionality on the parent molecule, and would thus be sufficiently efficient to allow the preparation of libraries of immobilized inhibitors, and thus to perform simultaneous structure-activity relationships on the complete proteome of the cell by affinity chromatography. Based on

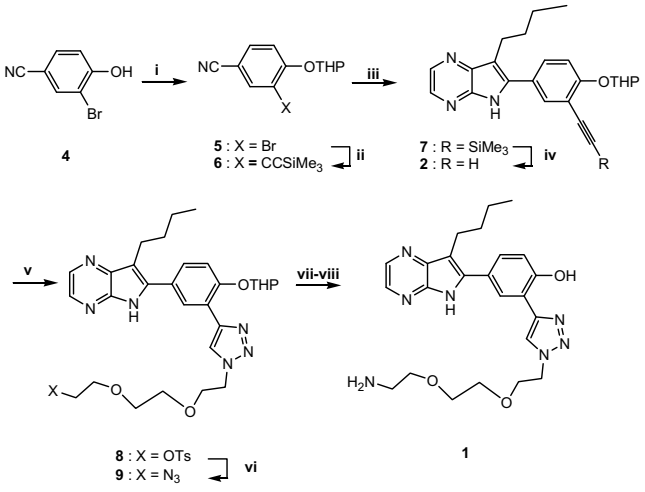


Figure 3. Synthesis of aloisine A analog **1** bearing a linker at the *C*-3′ position. Reagents and conditions: (i) DHP, PPTS, CH_2Cl_2 (57%) (ii) PPh_3, $PdCl_2(PPh_3)_2$, trimethylsilyl)acetylene, TEA, CuI, THF (77%); (iii) LDA, *n*-pentylpyrazine, THF, −40 °C; (iv) TBAF, THF (80%); (v) **3**, DIPEA, MeCN, CuI, rt (85%); (vi) NaN_3, DMF, 70 °C (77%); (vii) *p*-TSA, MeOH (84%); (viii) H_2, 10%Pd/C, MeOH, rt (64%).

previous SAR studies on aloisine,[12] we thus decided to explore some structural modifications, such as replacement of the 4′-hydroxyl for a chlorine (aloisine B), a methoxy, or a tetrahydropyranoxy, as well as the suppression of the butyl chain at C-7. To this aim, a small 'proof of concept' library of 3′-alkynyl aloisines was prepared according to Figure 4. 4-methoxybenzonitrile (**10**) was brominated by *ortho*-lithiation/bromination in the presence of LiTMP and $ZnCl_2$[26] to give an inseparable 15:85 mixture of 2- and 3-bromo-4-methoxybenzonitrile regioisomers **12a** and **12b**. Sonogashira coupling with (trimethylsilyl)acetylene on the mixture afforded, after purification, the desired regioisomer **14b** in a 47% yield over two steps, along with a small amount of the 2-ethynyl regiosiomer **14a**. The same strategy applied to the 4-chlorobenzonitrile (**11**) gave the 2-bromo-4-chorobenzonitrile regioisomer **13a** as the major product (ratio 80:20). Sonogashira coupling with (trimethylsilyl)acetylene yielded an unseparable mixture of **15a** and **15b**. The condensation step was performed using either methyl or pentylpyrazine. As before, reaction of the 3-alkynyl-4-methoxybenzonitrile **14b** in the presence of 2 equivalents of LDA followed by removal of the silyl protecting group afforded the desired pyrrolo[2,3-*b*]pyrazine cores **18** and **19** in 66% and 82% yields, respectively. Surprisingly, all attempts at condensation with the 2-alkynyl isomer **14a** failed, presumably due the steric hindrance of the acetylene group. Attempted condensation of the mixture of the 4-chlorobenzonitrile acetylenes **15a** and **15b** with pentylpyrazine under the same conditions yielded a complex mixture, of which only the de-halogenated pyrrolo[2,3-*b*]pyrazine **20** could be identified as a product.

Having in hand the acetylene-substituted pyrrolo[2,3-*b*]pyrazine compounds **2**, **18**, **19**, and **21** (the latter obtained from **2** by removal of the THP), we next turned our attention to their immobilization onto beads. In order to immobilize the compounds, commercially available 1,4-bis(2:3-epoxypropoxy)butane-derivatized agarose beads (Epoxy-Agarose, Aldrich) were reacted with sodium azide in water while maintaining the pH below 9 to provide the azido functionalized gel **22**. A corresponding azido-substituted gel **23** was prepared based on LCC-Reactospheres® beads, an amino-functionalized polymethyl methacrylate polymer with highly polar surface, which have the advantage of being resistant to a wide range of chemical conditions, in the event that further derivatization of the analogs on the gel would be desired. The alkynyl-aloisines **2**, **18**, **19**, and **21** were then immobilized under click coupling conditions[24,25] in the presence of CuI and DIPEA in acetonitrile to afford the corresponding immobilized aloisines **24**–**28** (Table 1).[27] The efficiency of the reaction was monitored qualitatively by visualization of the characteristic aloisine fluorescence on the beads under a UV lamp.

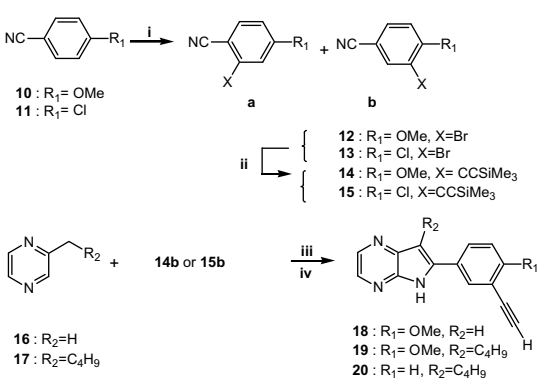
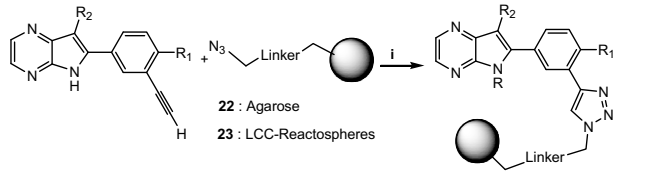


Figure 4. Synthesis of 3′-alkynylaloisines. Reagents and conditions: (i) LiTMP, $ZnCl_2$, Br_2, −70 °C; (ii) PPh_3, $PdCl_2(PPh_3)_2$, (trimethylsilyl)acetylene, TEA, CuI, THF (**14a**, 8% **14b**, 47%); (iii) LDA, THF, −40 °C; (iv) TBAF, THF (**18**, 66%; **1** 82%).

Table 1
Immobilization of the alkynyl aloisines



i : DIPEA, CuI, MeCN

Alkyne-Aloisine	R_1	R_2	Azido-Gel	Product
18	OMe	H	**22**	**24**
19	OMe	C_4H_9	**22**	**25**
2	OTHP	C_4H_9	**22**	**26**
21	OH	C_4H_9	**22**	**27**
21	OH	C_4H_9	**23**	**28**

The affinity chromatography assays were performed on porcine brain extracts using the different gels (see Supplementary data Fig. S1). While a detailed biological discussion of the results will be reported in due course,[14] a qualitative analysis of the results permits a few relevant observations: comparing the results using the compound **1** immobilized on cyanogen bromide-activated agarose with those of the gel **27** having essentially the same structure, although the capacity of the latter is lower (consistent with the lower degree of functionalization of the commercial epoxy sepharose compared to cyanogen-bromide activated agarose), the same target proteins are retained, and are not retained by the ethanolamine control. This demonstrates that the aloisine analogs were indeed immobilized by click chemistry to the azido-substituted Epoxy-Agarose gel. The reactosphere gel (**28**), although having the advantage of greater chemical stability, appears to bind the same target proteins, but also leads to more extensive non-specific binding. Comparison of the results obtained with the different gels (see Supplementary data Fig. S2) shows that it is in fact possible to obtain qualitative structure-activity relationships against the entire proteome by affinity chromatography. Although we have used a very limited library as a proof of concept, the synthesis of the precursors and the immobilization and affinity chromatography protocols are sufficiently simple to be applied to larger focused libraries.

In conclusion, we have described the preparation of immobilized forms of aloisine for selectivity screening by affinity chromatography, based on the incorporation of an acetylene group, followed by late stage immobilization, either in solution or in the solid state. This method represents an advantageous solution to the problem of incorporating hydrophilic and chelating polyethylene glycol chains that are often incompatible with the synthesis of the original compounds. This approach is efficient enough for immobilizing small libraries for selectivity optimization against complete cell extracts.

Acknowledgments

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Supplementary data

Affinity chromatography results with the immobilized aloisines.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.11.051.

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19. *Typical procedure for the Sonogashira reaction*: Synthesis of compound **6**: PPh_3 (61 mg, 0.23 mmol), $PdCl_2(PPh_3)_2$ (378 mg, 0.54 mmol), and 1.1 g of compound **5** (3.9 mmol., prepared by THP protection of the 4-hydroxybenzonitrile) were dried for 30 min in a schlenk tube. 10 mL of THF, 2.7 mL of TEA (19.5 mmol), and 1.1 mL of (trimethylsilyl)acetylene (7.8 mmol) were added to the mixture. After 30 min, CuI (0.05 equiv) were added to the solution. The reaction mixture was stirred at rt under an atmosphere of argon for 12 h. The solvent was removed under vacuum and the solid was filtered off. The crude product was purified by flash chromatography over silica gel and was eluted with PE/EA (85/15). Yield 900 mg (77%). ^{1}H NMR ($CDCl_3$): δ (ppm) 7.68 (1H, d, J = 2.1 Hz); 7.50 (1H, dd, J = 8.7 Hz, J = 2.2 Hz); 7.12 (1H, d, J = 8.7 Hz); 5.60 (1H, s); 3.80 (1H, dt, J = 2.6 Hz, J = 11.0 Hz); 3.60 (1H, m); 2.05–1.60 (6H, m); 0.25 (9H, s). HRMS calcd for $C_{17}H_{21}NO_2Si$: 300.1420; found: 300.1420.
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21. *Typical procedure*: Synthesis of compound **7**: *n*-pentylpyrazine (175 mg, 1.16 mmol) was added to a freshly prepared solution of LDA (2.32 mmol in 4 ml of THF-hexane) at −40 °C under argon. After 30 min, compound **6** (180 mg, 0.58 mmol) in THF (1 mL) was added, and the solution was stirred for 30 min at −40 °C and for 18 h at rt, then hydrolyzed with a saturated aqueous solution of NH_4Cl. After extraction with ethyl acetate, the organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude product was purified by flash chromatography over silica gel and was eluted with EA/PE (2/8) to yield a mixture of compound **7** and **2**, from which a pure fraction of **7** was characterized. ^{1}H NMR: (300 MHz, $CDCl_3$): δ (ppm) 12.60 (1H, s); 8.40 (1H, d, J = 2.8 Hz); 8.20 (1H, d, J = 2.7 Hz); 7.90 (1H, d, J = 2.2 Hz); 7.68 (1H, dd, J = 8.8 Hz, J = 2.4 Hz); 7.25 (1H, d, J = 8.8 Hz); 5.68 (1H, br s); 4.00 (1H, m); 3.68 (1H, m); 2.99 (2H, t, J = 7.7 Hz); 2.10–1.60 (8H, m); 1,40 (2H, m); 0,90 (3H, t, J = 7.3 Hz); 0.25 (9H, s). HRMS calcd for $C_{26}H_{33}N_3O_2Si$: 448.2420; found: 448.2423.
22. *Typical procedure for the click reaction*: Synthesis of compound **8**. To a stirred solution of compound **2** (320 mg, 0.85 mmol) in MeCN (10 mL) were added 280 mg of compound **3** (0.85 mmol). After 20 min at rt, 445 µL of DIPEA (2.55 mmol) and a catalytic amount of CuI (~0.05 equiv) were added, the resulting mixture was stirred at room temperature for 14 h and the reaction was stopped by adding 2 mL of water. The resulting solution was extracted with ethyl acetate, the organic phase washed with NaCl, dried over Na_2SO_4, and concentrated under vacuum. The crude product was purified by flash chromatography over silica gel and was eluted with EA/PE (95/5). Yield 520 mg (86%). ^{1}H NMR: (300 MHz, $CDCl_3$): δ (ppm) 11.10 (1H, br s); 8.72 (1H, d, J = 2.1 Hz); 8.40–8.30 (3H); 8.22 (1H, d, J = 2.9 Hz); 7.72 (2H, d, J = 7.8 Hz); 7.65 (1H, dd, J = 8.6 Hz, J = 2.1 Hz); 7.42 (1H, d, J = 8.9 Hz); 7.25 (2H, d, J = 7.8 Hz); 5.60 (1H, br s); 4.62 (2H, t, J = 4.9 Hz); 4.20 (2H, t, J = 5.0 Hz); 3.90 (2H, t, J = 5.1 Hz); 3.83 (1H, m); 3.70 (1H, m); 3.62–3.58 (6H, m); 3.05 (2H, t, J = 7.7 Hz) 2.43 (3H, s, CH3Ts); 2.00–1.65 (8H, m); 1.45 (2H, tq, J = 7.5 Hz); 0.88 (3H, t, J = 7.4 Hz). HRMS calcd for $C_{36}H_{44}N_6O_7S$: 705.3070; found: 705.3068.
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27. (a) Azido substituted Agarose: Commercial Epoxy-Agarose (Aldrich 1 g, 20–40 mol/mL) was suspended in aqueous sodium azide (6 mL, 10 equiv) and stirred by rotation at 50 °C, while maintaining the pH below 9 by addition of aliquots of saturated aqueous solution of NH_4Cl. After 8 h, the resin was filtered and washed with water. (b) Azido substituted Reactospheres: The azido diglycolic acid PEG n7, MW 554.6 (500 mg, LCC) is attached on the amino PMMA LCC-Reactospheres® A310 resin (2.5 g) in DMF (4 mL) by adding EDCI (180 mg, 2 equiv) and HOBt (400 mg, 3 equiv). The mixture is allowed to react overnight at room temperature. The resin is then filtered and washed with MeOH/DMF, MeOH, DCM, and DMF. The synthesis step is repeated twice.(c) *Typical conditions for the on-gel click reaction:* The azido substituted agarose (1 mL) was suspended in acetonitrile (3 mL) containing **2** (0.03 mmol), DIPEA (5 equiv to the alkyne), and a catalytic amount of CuI (~0.05 equiv). The mixture was stirred by rotation overnight. The resin was filtered and washed thoroughly with MeOH, DMF, CH_2Cl_2, and 0.2 M EDTA to provide **26** as a white, UV-fluorescent gel, indicating the presence of immobilized **2**. Further IR measurements indicated the disappearance of the azido group and the affinity chromatography results provided in the supplementary material demonstrate the presence of the immobilized aloisines.