Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 14 (2004) 4203–4206

Copper dipicolinates as peptidomimetic ligands for the Src SH2 domain

Boris Schmidt,[a,*] Jan Jiricek,[a] Alexander Titz,[a] Guofeng Ye[b] and Keykavous Parang[b]

[a]*Institute for Organic Chemistry and Biochemistry, Darmstadt Technical University, Petersenstr. 22, D-64287 Darmstadt, Germany*
[b]*Department of Biomedical Sciences, University of Rhode Island, 41 Lower College Road, Kingston, RI 02881-0809, USA*

Received 27 April 2004; revised 8 June 2004; accepted 8 June 2004
Available online 2 July 2004

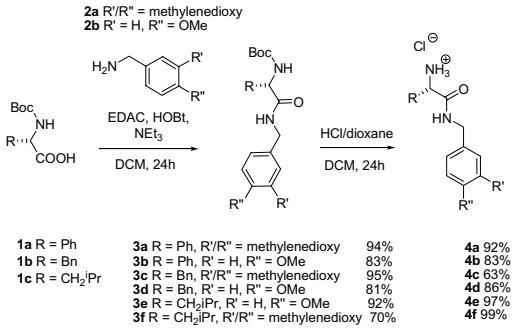
Abstract—The introduction of copper chelates into peptide mimetics creates the Src SH2 binding ligands and paramagnetic complexes suitable for EPR studies of peptide protein interactions. The dipicolinic acid was attached to SH2 domain targeting fragments by two different linkers.

1. Introduction

The complexation of transition metals by amino acids is a well established strategy for controlling the shape and the activity of peptides and proteins.[1,2] Furthermore, paramagnetic complexes are of interest for EPR and NMR studies of specifically labelled proteins.[3–5] In our ongoing search for suitable metal complexes,[6–8] the paramagnetic copper(II) complexes of 4-substituted 2,6-pyridine dicarboxylates (**11a–d**), derived from chelidamic acid (**5**), were investigated here. Chelidamic acid as a tridentate chelator displays a high affinity for binding to Cu(II) ($\log K_1 = 12.2$, $\log K_2 = 9.9$).[9] The copper/chelidamic acid 1:2 complex is easily transferred into 1:1:1 complexes with coordinating amines. The affinity and selectivity of metal complexes in aqueous solutions are not the only criteria for the planned ESR studies. Additionally, the resulting complexes have to be sufficiently stable in the presence of proteins to serve as tools for investigating complex biological systems. The incorporation of dipicolinic copper and silver complexes into double stranded DNA on duplex formation has been previously reported,[10,11] supporting our notion that this approach could be also used for designing peptidomimetic complexes and designing ligands for interaction with proteins. Small peptide fragments were investigated here as potential ligands for binding to Src homology 2 (SH2) domain of Src tyrosine kinase and to explore the accessibility and stability of the complexes prior to their incubation with the target proteins. Src is a member of nonreceptor Src family kinases that contain an N-terminal unique domain followed by two Src homology domains, SH3 and SH2, a kinase domain, and a short C-terminal regulatory peptide segment. SH2 domains are modules of approximately 100 amino acids that have evolved to recognize and bind specifically to tyrosyl-phosphorylated sequences located on proteins in response to extracellular signals.[12–15] Enhanced Src tyrosine kinases activity has been directly linked to T-cell activation, mitogenesis, differentiation, and oncogenesis.[16] Ligands having the ability to disrupt cellular signal transduction pathways by antagonizing SH2 domain dependent protein–protein interactions provide possible therapeutic agents.[17] The Src SH2 domain has two major interaction sites that is, the hydrophilic phosphotyrosine binding pocket (P site) and the hydrophobic pocket. The phosphotyrosine residue of phosphopeptide ligands such as pYEEI is buried in the deep positively charged pocket (P site).[18] The design of SH2 inhibitors has focused on peptidomimetics modifications of cognate peptide sequences,[19] conformationally constrained peptides,[20] and replacement of phosphotyrosine residues by various types of pTyr mimics.[21] In the search for pTyr mimetic peptides, which are stable to phosphatases, several dipicolinic acids, dipicolinates, and copper dipicolinates were investigated and compared for binding affinity to the Src SH2 domain as potential ligands for possible interaction with P site. Several hydrophobic peptide fragments, **4a–f**,

Keywords: SH2 domain; Copper complex; ESR; Peptide mimetic.
* Corresponding author. Tel.: +49-615-1163075; fax: +49-615-11632-78; e-mail: schmibo@oc.chemie.tu-darmstadt.de

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.06.018

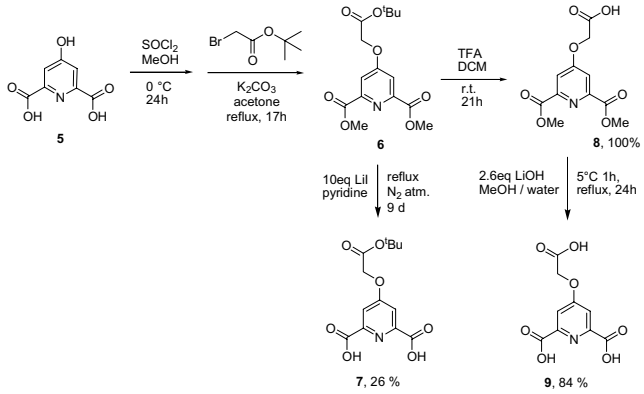


Scheme 1. Synthesis of SH2 directed peptide fragments.

which may improve the binding affinity through interaction with hydrophobic pocket of the Src SH2 domain, were linked to dipicolinate derivatives. Src kinases are held on the cytoplasmic side of the plasma membrane, bound partially both to a transmembrane protein receptor and to lipid chains that intercalate in the cell membrane, thus it is expected that metal complexes do not need completely to cross membranes to reach the target in cell-based assays.

Scheme 1 shows the procedure for the preparation of SH2 directed peptide fragments (**4a**–**f**). Reactions of Boc-protected amino acids (**1a**–**c**), with benzylamine substituted derivatives, **2a** and **2b**, in the presence of EDAC (1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide·HCl) and HOBt (*N*-hydroxybenzotriazole·H_2O) as coupling reagents in dichloromethane (DCM) followed by subsequent deprotection of Boc group by HCl in dioxane yield **4a**–**f**, which were used as hydrochloride salts in reactions with the carboxylic acid **8** (Scheme 3).

Scheme 2 shows the procedure for the synthesis of dimethyl ester derivative of dipicolinate **8** and several methods[10,11] for the selective deprotection of the methyl esters in the presence of the ^{t}butyl ester. The chelidamic acid **5** was converted into the dimethylester in 81% yield according to previously reported procedure.[8] The alkylation of the dimethyl ester with ^{t}butyl bromoacetate was accomplished by refluxing in acetone over potassium carbonate to provide the intermediate triester **6**.



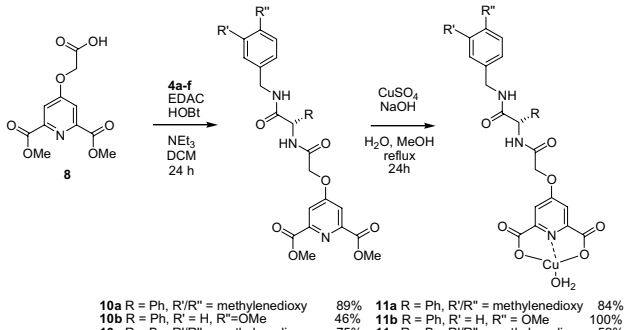
Scheme 2. Synthesis and selective deprotection of dipicolinate ester derivatives.

Lithium iodide in refluxing pyridine selectively deprotected dimethyl esters to yield **7**, but the reaction was rather slow and suffered from both poor turnover and decomposition. Only 26% of the desired product **7** was isolated after 9 days. On the other hand, selective deprotection of the ^{t}butyl ester was accomplished by treatment of isolated crude product **6** with trifluoroacetic acid in dichloromethane to yield the monocarboxylic acid **8** in 63% overall yield starting from **5** and 100% yield from **6**. The dimethyl ester **8** was converted to tricarboxylic acid derivative **9** in 84% yield in the presence of lithium hydroxide.

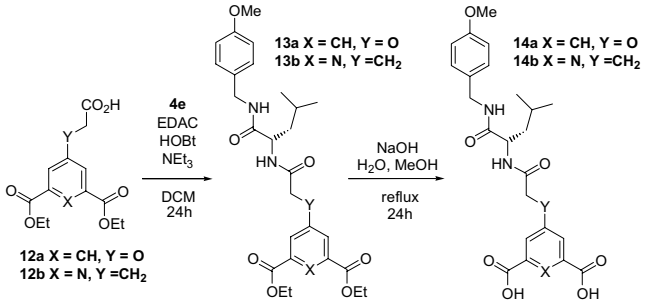
Scheme 3 shows the general protocol for the preparation of copper(II) complexed peptides, **11a**–**d**. The reaction of **8** with **4a**–**f** in the presence of EDAC/HOBt as coupling reagents provided **10a**–**f** in 46–89% yield. The final hydrolysis of the methyl esters **10a**–**d** and complexation proceeded smoothly in refluxing aqueous methanol containing copper sulfate pentahydrate (1 equiv) and sodium hydroxide (2 equiv). The color of the reaction mixture turned dark blue upon addition of the sodium hydroxide, but faded to a light blue clear solution within 24 h. Cooling of the reaction mixture to room temperature provided the desired copper complexes **11a**–**d** as amorphous precipitates upon filtration. These compounds (**11a**–**d**) turned into a dark turquoise color after filtration through a Buchner funnel, but regained their light blue color upon extended drying at low pressure.

Although the pH of the reaction mixture reached to 5 at the end of the reaction, most of the hydrolysis of methyl esters took place at a rather basic pH. The most active compound **11d** was not prone to racemization under these conditions. The solutions of **11a** and **11b** were investigated at higher concentrations, which favor dimeric species, and displayed no signal doubling in the ^{1}H NMR. This experiment ruled out the presence of diastereomeric 2:1 complexes deriving from substantial racemization.

A similar strategy was used to synthesize dicarboxylic acids **14a** and **14b** from the triacid derivatives **12a**,**b** (Scheme 4).



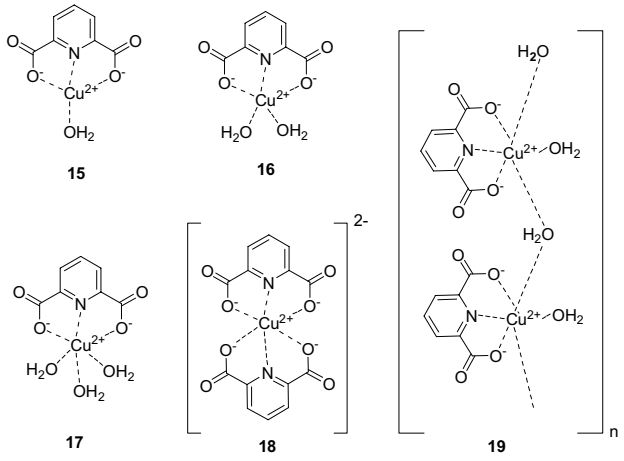
Scheme 3. Synthesis of copper(II) complexed peptides.



Scheme 4. The synthesis of dicarboxylic acid derivatives through *C*- or *O*-attachment with peptide fragments.

Several diverse structures for copper dipicolinates (**15**–**19**, Scheme 5) have been previously reported.[22–28] The formation of these structures depends on the stoichiometry and the detailed conditions of the synthesis. Most of the described complexes were characterized in the solid state or in homogenous solution in the absence of additional ligands which may not be relevant to conditions used in the synthesis of copper complexes **11a**–**d**. As the progress of our program depends on the structures in buffered, aqueous solutions, we have made no attempts to elucidate the number of water in the isolated precipitates as anticipated by structures **15**–**19**. Further studies using EPR techniques are now underway to obtain further information about the anticipated 1:1 complex in solution. For this purpose the hyperfine interaction between the paramagnetic copper center and nuclear spins of the chelate, as for example copper–nitrogen and copper–hydrogen, are investigated. Multipulse EPR and ENDOR techniques are invoked, of the broad unresolved EPR spectrum.

The peptidomimetics obtained were stable in air or moisture at neutral pH allowing their incubation with target proteins for binding assays. The binding affinity for several derivatives to the Src SH2 domain is reported in Table 1 and is characterized by a at least 23-fold higher affinity of the copper complexes versus their parent dimethylesters and free dicarboxylic acid **14a**,



Scheme 5. Copper species in aqueous solution.

Table 1. Binding affinity of compounds for the Src SH2 domain

Compounds	IC_{50} (μM)[a]
10a	714
10b	NA[b]
10c	396
10d	663
10e	713
11b	20
11d	17
14a	864

[a] IC_{50}: The concentration that inhibits the binding of the fluorescent probe (GpYEEI) to the Src SH2 domain by 50%. Values are means of three experiments with a standard deviation of less than ±5%.

[b] NA = no significant inhibition was observed at maximum concentration tested 500 μM).

suggesting that hydrophobic interaction is not contributing significantly to the binding affinity of these compounds. Additionally, two carboxylic acids groups in compound **14a** are not properly oriented to interact with positively charged amino acids in the Src SH2 domain pocket and/or their negative charges do not mimic the phosphate group of phosphopeptides such as pYEEI. Whether significant increase in the binding affinity by copper complex compounds, **11b** and **11d**, is due their polarization and/or proper orientation for specific interactions with the positively charged amino acids in the phosphotyrosine binding pocket of the Src SH2 domain is subject to ongoing NMR and EPR investigations. In conclusion, copper complexes of dipicolinic acid derivatives exhibited significantly higher binding affinity to the Src SH2 domain than the corresponding analogs without copper. This approach could be used with other optimal aromatic dicarboxylic acids to design novel pepidomimetic SH2 domain inhibitors that do not have a phosphate group.

2. Materials and methods

Expression and purification of Src SH2 domain: A culture of *E. coli* (strain TGI) containing pGEX-2T in YT broth with 100 μg/mL ampicillin was prepared and allowed to incubate with shaking at 37 °C overnight (Innova 2000 Platform Shaker, New Brunswick Scientific, 200 rpm). A fresh 300 mL YT broth containing 100 μg/mL ampicillin was mixed with the overnight culture, induced with 0.2 mM isopropylthio-galactoside (IPTG), and incubated at room temperature with shaking for 5 h. The cells were harvested by centrifugation at 5000*g* for 5 min. The supernatant was discarded and the cell pellet resuspended in 5 mL 1X PBS buffer (0.1% β-mercaptoethanol). The cell lysis was carried out via sonication (three separate 10 s bursts, with 30 s incubations on ice in between each burst). The lysate was centrifuged at 15,000*g* for 20 min and the supernatant was added to a 4 μM slurry of glutathione-S-transferase (GST) in 1X PBS buffer (0.1% β-mercaptoethanol). The protein was released from the GST affinity column by the addition of 5 mL reduced GST (0.1% β-mercaptoethanol) and detected by 5X Bradford reagent. The concentration of

protein was determined by reading optical density employing a Pharmacia Biotech Ultrospec 2000 UV/VIS spectrophotometer using bovine serum albumin (BSA, 1.0 mg/mL) as a standard.

3. Src SH2 domain binding assay

All peptides were tested as competitors against the fluorescent probe for binding affinity to the Src SH2 domain using a fluorescent polarization (FP) competitive binding assay as described below. FP was measured at 25 °C in a disposable glass tube (volume, 600 L) using a Perkin Elmer LS 55 Luminescence spectrometer equipped with a FP apparatus. The excitation and emission wavelengths were set at 485 and 535 nm, respectively. For the competition assay, final concentrations of 750 nM SH2, 80 nM fluorescent probe, phosphate buffer (20 mM, pH 7.3, 100 mM NaCl, 2 mM DTT, 0.1% BSA), water, and various concentrations of each competitor compound were used. The assay was designed so that the concentration of competing peptide in any tube was doubled relative to the previous tube. The order of addition to each 600 μL glass tube was: (i) buffer, (ii) water, (iii) fluorescent probe, (iv) SH2 domain, and (v) competitor peptide. A blank control with the Src SH2 domain, but without a peptide, and a background control without both the Src SH2 domain and peptide were used. The inhibition percentage (IP) of fluorescent probe binding to the Src SH2 domain by the sample was calculated by the following equation:

$$\left[1 - \frac{FP_{blk} - FP_s}{FP_{blk} - FP_{bgd}}\right] \times 100$$

where FP_{blk} is the fluorescent polarization value of the blank control; FP_s is the fluorescent polarization value of the sample (peptide); and FP_{bgd} is the fluorescent polarization value of the background control. The inhibition percentages of the various concentration of the assayed peptide were plotted and the IC_{50} value (a concentration that inhibits the binding of the fluorescent probe to the Src SH2 domain by 50%) was calculated using a CurveExpert 6.0 software. The reported IC_{50} values are the mean of three separate determinations with a standard deviation of less than ±5%.

Acknowledgements

We thank the Fonds der Chemischen Industrie and the DFG for support of this work. We also acknowledge the financial support from US National Center for Research Resources, NIH, Grant number 1 P20 RR16457.

References and notes

1. Kratz, F. Met. *Complexes Cancer Chemother.* **1993**, 391–429.
2. Pickart, L. *Chem. Biochem. Amino Acids, Pept., Proteins* **1982**, *6*, 75–104.
3. Basosi, R.; D'Amelio, N.; Gaggelli, E.; Gaggelli, N.; Maccotta, A.; Pogni, R.; Valensin, D.; Valensin, G. *Curr. Top. Pept. Protein Res.* **2001**, *4*, 17–35.
4. Taiwo, F. A. *Spectroscopy (Amsterdam, Netherlands)* **2003**, *17*, 53–63.
5. Banci, L.; Pierattelli, R.; Vila, A. J. *Adv. Protein Chem.* **2002**, *60*, 397–449 (Copper-Containing Proteins).
6. Muller, G.; Schmidt, B.; Jiricek, J.; Buenzli, J. C. G.; Schenk, K. J. *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **2003**, *59*, o353–o356.
7. Muller, G.; Schmidt, B.; Jiricek, J.; Hopfgartner, G.; Riehl, J. P.; Buenzli, J.-C. G.; Piguet, C. *J. Chem. Soc., Dalton Trans.* **2001**, 2658–2665.
8. Schmidt, B.; Ehlert, D. K. *Tetrahedron Lett.* **1998**, *39*, 3999–4002.
9. Smith, R. M.; Martell, A. E. In *Critical Stability Constants Constants*; Plenum: New York, 1975; Vol. 1.
10. Meggers, E.; Holland, P. L.; Tolman, W. B.; Romesberg, F. E.; Schultz, P. G. *J. Am. Chem. Soc.* **2000**, *122*, 10714–10715.
11. Zimmermann, N.; Meggers, E.; Schultz, P. G. *J. Am. Chem. Soc.* **2002**, *124*, 13684–13685.
12. Beaulieu, P. L.; Cameron, D. R.; Ferland, J.-M.; Gauthier, J.; Ghiro, E.; Gillard, J.; Gorys, V.; Poirier, M.; Rancourt, J.; Wernic, D.; Llinas-Brunet, M.; Betageri, R.; Cardozo, M.; Hickey, E. R.; Ingraham, R.; Jakes, S.; Kabcenell, A.; Kirrane, T.; Lukas, S.; Patel, U.; Proudfoot, J.; Sharma, R.; Tong, L.; Moss, N. *J. Med. Chem.* **1999**, *42*, 1757–1766.
13. Buchanan, J. L.; Vu, C. B.; Merry, T. J.; Corpuz, E. G.; Pradeepan, S. G.; Mani, U. N.; Yang, M.; Plake, H. R.; Varkhedkar, V. M.; Lynch, B. A.; MacNeil, I. A.; Loiacono, K. A.; Tiong, C. L.; Holt, D. A. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2359–2364.
14. Cody, W. L.; Lin, Z.; Panek, R. L.; Rose, D. W.; Rubin, J. R. *Curr. Pharm. Des.* **2000**, *6*, 59–98.
15. Shakespeare, W. C. *Curr. Opin. Chem. Biol.* **2001**, *5*, 409–415.
16. Biscardi, J. S.; Tice, D. A.; Parsons, S. *J. Acv. Cancer Res.* **1999**, *76*, 61–119.
17. Sawyer, T. K. *Biopolymers* **1998**, *47*, 243–261.
18. Songyang, Z.; Shoelson, S. E.; Chaudhuri, M.; Gish, G.; Pawson, T.; Haser, W. G.; King, F.; Roberts, T.; Ratnofsky, S.; Lechleider, R. J.; Neel, B. G.; Birge, R. B.; Fajardo, J. E.; Chou, M. M.; Hanafusa, H.; Schaffhausen, B.; Cantley, L. C. *Cell* **1993**, *72*, 767–778.
19. Park, J.; Fu, H.; Pei, D. *Biochem.* **2003**, *42*, 5159–5167.
20. Nam, N.-H.; Ye, G.; Sun, G.; Parang, K. *J. Med. Chem.* **2004**, *47*, 3131–3141.
21. Nam, N.-H.; Pitts, R. L.; Sun, G.; Sardari, S.; Tiemo, A.; Xie, M.; Yan, B.; Parang, K. *Bioorg. Med. Chem.* **2004**, *12*, 779–787.
22. Faucherre, J.; Petitfaux, C.; Charlier, B. *Bull. Soc. Chim. Fr.* **1967**, 1091–1098.
23. Koman, M.; Moncol, J.; Hudecova, D.; Dudova, B.; Melnik, M.; Korabik, M.; Mrozinski, J. *Pol. J. Chem.* **2001**, *75*, 957–964.
24. Mao, L.; Wang, Y.; Qi, Y.; Cao, M.; Hu, C. *J. Mol. Struct.* **2004**, *688*, 197–201.
25. Mojumdar, S. C.; Melnik, M.; Jona, E. *J. Anal. Appl. Pyrol.* **2000**, *53*, 149–160.
26. Murtha, D. P.; Walton, R. A. *Inorg. Chim. Acta* **1974**, *8*, 279–284.
27. Sileo, E. E.; Quinteros-Rivero, M. F.; Rivero, B. E.; Rigotti, G.; Blesa, M. A. *Solid State Ionics* **1994**, *73*, 153–159.
28. Sorensen, J. A.; Thompson, L. C.; Glass, G. E. *Anal. Chem.* **1985**, *57*, 1087–1091.