Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1665–1669

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Selective Inhibition of Src SH2 by a Novel Thiol-Targeting Tricarbonyl-Modified Inhibitor and Mechanistic Analysis by $^{1}H/^{13}C$ NMR Spectroscopy

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Received 9 February 2001; accepted 12 April 2001

Dedicated to Professor Michael P. Cava, Robert Ramsay Professor of Chemistry, UAL, Tuscaloosa, AL, on his 75th birthday

Abstract—Detailed analysis of Src SH2 binding by peptides containing a novel tricarbonyl-modified pTyr moiety is described. We envisaged that Src SH2 selectivity might be obtained by exploiting the thiol group of Cys188 present in the pTyr binding pocket of the protein at the βC3 position. Peptidyl as well as non-peptidyl compounds **1**–**4** possessing a 4-α,β-diketoester-modified pTyr mimic exhibited micromolar affinity to Src SH2. Furthermore, these tricarbonyl compounds were selective for Src SH2 to the extent they showed no significant affinity for either Cys188Ser or Cys188Ala Src SH2 mutants. Upon closer examination of the binding of these tricarbonyls to Src SH2 using NMR of ^{13}C-labeled compounds (**6a**, **6b**, and **6c**), we found that after the initial binding event the molecule disproportionated in a 'retro-Claisen' fashion to provide benzoic acid **16** and, following hydrolysis of the methyl ester **17**, the hemiketal adduct of glyoxalic acid **18**.

The Src family of tyrosine kinases play an important role in regulating intracellular signalling. The SH2 domain binds to pTyr-containing proteins and peptides in a sequence dependent manner,[1] which provides the basis for differentiation of the phosphorylation/dephosphorylation events surrounding a multitude of intracellular signalling pathways.[2] This intricate role of SH2 domains coupled with the postulated involvement of the nonreceptor tyrosine kinase Src in various diseases provides an incentive to develop Src SH2 binding small molecules as therapeutic agents to inhibit the signalling activity associated with diseases such as cancer and osteoporosis.[3] The SH2 domain of Src differs from other closely related SH2 domains in that it contains a unique cysteine (Cys188) located in the pTyr binding pocket. A Cys at the βC3 position is unique to Src, even within the Src family, as all the other family members possess Ser in this position. Careful examination of the Src SH2 crystal structure reveals that this Cys associates with the imidazole of an adjacent His, which should serve to enhance the nucleophilicity of the Cys. Modeling predicted a 3.9 Å gap into which a small electrophile appropriately positioned might engage the thiol to enhance binding affinity and selectivity. Synthesis of reversible, high affinity ligands for Cys-proteases in which the active site contains a reactive thiol has been reported earlier.[4] Although it is unlikely that the thiol of Cys188 is fully ionized at neutral pH, the concentration of positively-charged residues in the pocket is likely to decrease the pK_a. In fact, in an elegant study Singer and Forman-Kay[5] determined the p*K*s of His57 and pTyr in a PLCγ-1 C-terminal SH2 domain-pY1021 complex to be significantly reduced due to the proximity of these groups to the highly charged pTyr binding pocket. Thus, a rational approach to the design of Src specific ligands would involve engineering a pTyr mimic with an appropriately positioned electrophile that could covalently engage the reactive Cys. The synthesis of many such Cys-targeted ligands is reported in the literature.[5–9] Critical to the success of this approach for discovery of viable drug candidates is that the electrophilic moiety of the ligand be highly selective and not reactive to other

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0960-894X/01/$ - see front matter
PII: S0960-894X(01)00269-4

nucleophiles, particularly other thiols, or alternatively, that the interaction with the thiol be reversible. The present study shows a tricarbonyl-modified pTyr mimic provides such interactions with Cys188 of Src SH2 (Fig. 1a and b).

Hatada et al.[10] determined the crystal structure of Src SH2 at 1.5 Å resolution and found that the protein contained a well complexed citrate ion in the pTyr binding pocket, when crystallized from citrate buffer. The citrate was held by a number of hydrogen bonds to the protein including both side-chain and main-chain atoms. A vicinal tricarbonyl moiety could function as a phosphate mimic when superimposed with the citrate structure in the pTyr binding pocket of SH2 domain. Independently, another group also claimed that the tetrahedral center of phenyl phosphate resembles the hydrated form of the tricarbonyl compound.[11] Molecular modeling reported earlier[12] also suggested that the polar tricarbonyl moiety may occupy the phosphate binding pocket of SH2 domains in a manner similar to that of phosphonates and malonates.[13] None of these papers has delineated the fragile nature of the tricarbonyls or the molecular basis of their mechanistic selectivity. Here, we report our synthesis of the tripeptides containing the tricarbonyl moiety and their selective interaction with the Cys188 of Src SH2. Furthermore we describe the mechanistic details of decompositon of the tricarbonyl moiety that involve a glyoxalic acid intermediate by analysis of the ^{13}C-labeled tricarbonyl compounds **6a–c** by NMR.

To test the above hypothesis, *p*-tricarbonyl-substituted-l-phenyl alanine containing tetrapeptides **1** and **2** were synthesized according to Scheme 1 using the earlier reported method of vicinal tricarbonyl synthesis by Wasserman and others.[11–14] Compounds **1** and **2** were synthesized from a common intermediate **7**, which was prepared from *N*-Boc-l-Tyr-OBn. Palladium mediated carbonylation of the triflate **7**[15] followed by hydrolysis of the TMSE group with TBAF gave compound **8**, which was converted to the protected tricarbonyl compound **9** using the Wasserman conditions.[14] Hydrogenolysis of the benzyl ester **9**, followed by removal of the Boc group with TFA and reprotection of this amine with acylimidazole in DME gave **10.** Coupling of the carboxylic acid **10** with the respective amines containing protected tripeptide $R'NH_2$ gave **11a** and **11b**. Oxidative deprotection of the protected tricarbonyl moiety by ozonolysis in methanol gave **12a** and **12b**. Deprotection of the benzyl ether of **12a** gave **1** and deprotection of the *t*-butyl ester of **12b** gave **2**. Compound **6** was synthesized from benzoic acid.[14] In a similar manner **3** and **4** were synthesized using Scheme 2, starting from 4-iodo benzoic acid TMSE ester **13** for **3** and starting from 3-iodobenzoic acid TMSE ester for **4** (not shown in Scheme 2).

Compounds **1–6** (Schemes 1 and 2 and Fig. 2) as well as the tetrapeptide Ac-pYEEI-NH_2 were analyzed in the BIAcore binding assay[16] against wild-type Src as well as Cys188Ala Src SH2 mutant. Table 1 shows the IC_{50} values for these compounds against both these proteins. All compounds exhibited a good affinity for wild-type Src SH2 and were selective for Src SH2 relative to Fyn SH2[17] as well as Cys188Ser[17] and Cys188Ala Src SH2 mutants. They were reversible, suggesting that the electophilic carbonyl engaged the Cys of Src SH2. Ac-pYEEI showed similar affinity to both wild-type Src as well as Cys188Ala Src SH2 to further confirm the selectivity of the tricarbonyls for Src SH2. Similarly, the simple *p*-carboxy substituted tripeptide **5** did not show any selectivity between wild-type Src SH2 and Cys188Ala Src SH2. Binding affinities of these compounds were

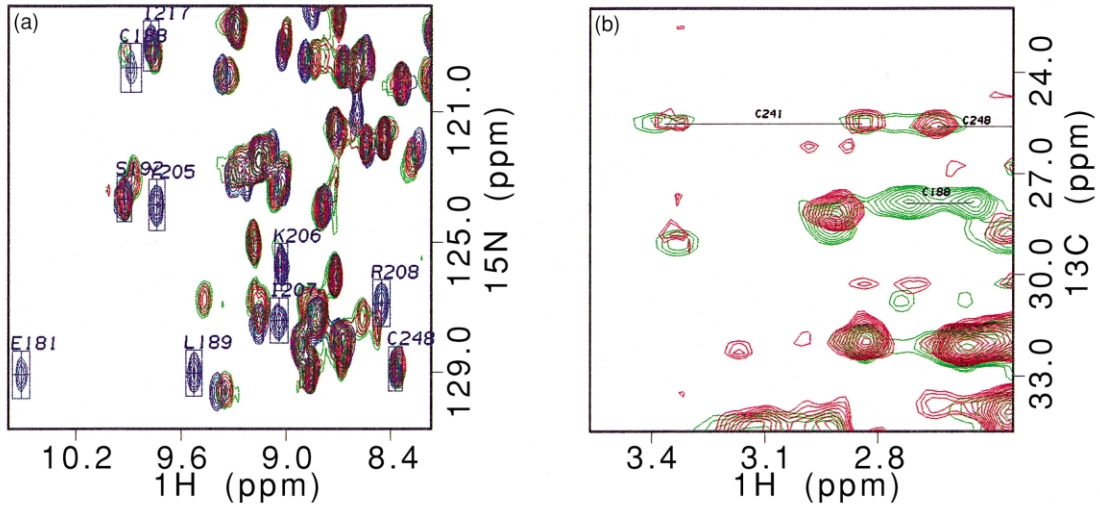
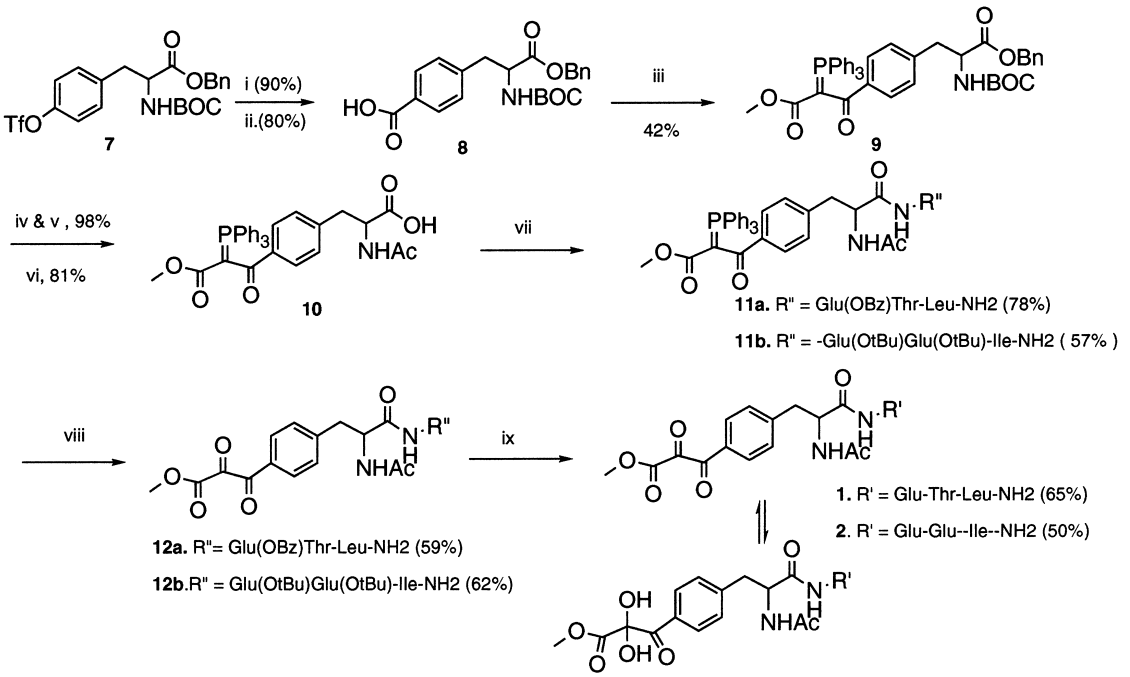
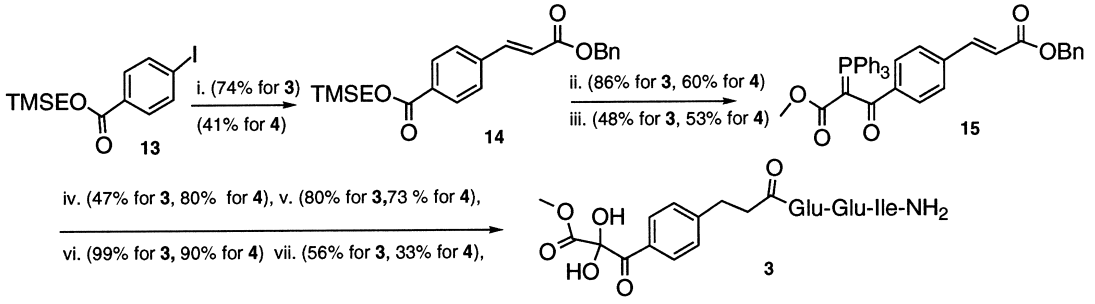


Figure 1. (a) A portion of ^1H–^{15}N HSQC spectrum of ^{15}N labeled Src SH2 complexed with **6a** and recorded at different time intervals is shown. The spectrum recorded within 1 h of complex formation is shown in blue and those recorded at 24 and 72 h intervals are shown in red and green, respectively. The horizontal and vertical axes represent ^1H and ^{15}N chemical shifts, respectively. The cross peaks shown in this portion represent the backbone HN/N of the marked residues of the ^{15}N labeled protein. (b) A portion of ^1H–^{13}C HSQC spectrum of double (^{15}N, ^{13}C) labeled Src SH2 complexed with **6c** and recorded at 30 h shown in red is compared with uncomplexed protein shown in green. The horizontal and vertical axes represent ^1H and ^{13}C chemical shifts, respectively. The marked peaks are arising from the $Cys C_\beta$–H_β resonances. Each cysteine is expected to give two cross peaks in this spectrum.



Scheme 1. (i) $Pd(OAc)_2$, dppp, CO, TMSE, Et_3N, 80 °C, DMSO, 2 h; (ii) Bu_4NF, THF, rt, 5 h; (iii) PPh_3 =CHCOOMe, EDC, THF, DMAP, rt, 28 h; (iv) MeOH, Pd/C, H_2, rt, 30 min; (v) TFA/CH_2Cl_2, rt, 30 min; (vi) *N*-acylimidazole, DME, Et_3N, rt, 18 h; (vii) EDC/HOBt,CH_2Cl_2/DMF, $R''NH_2$, rt, 4.5 h; (viii) O_3, MeOH, −78 °C to rt, 30 min; (ix) For **12a**, MeOH, Pd/C, H_2, rt, 16 h: For **12b.** TFA/$CH_2CH_2Cl_2$ (0.5% water), rt, 30 min.



Scheme 2. (i) $PdCl_2$, CH=CHCOOBn, $Bu_4NCl{\cdot}6H_2O$, DMF, 80 °C, 2.5 h; (ii) Bu_4NF, THF, rt, 5 h; (iii) PPh_3=CHCOOMe, EDC, THF, DMAP, rt, 28 h; (iv) MeOH, Pd/C, H_2, rt, 30 min; (v) EDC, HOBt, CH_2Cl_2/DMF, $R''NH_2$, rt, 4.5 h; (vi) O_3, MeOH, −78 °C to rt, 30 min; (vii) For **3**, TFA/CH_2Cl_2 (0.5% water), rt, 30 min; for **4**, MeOH, Pd/C, H_2, rt, 16 h.

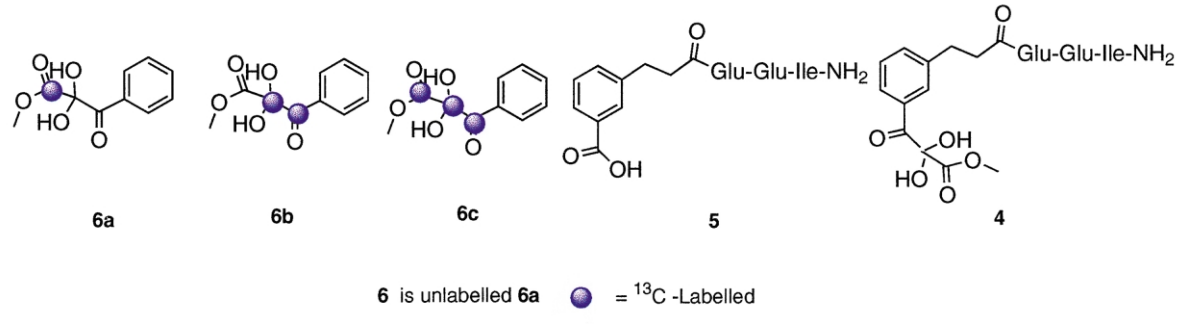


Figure 2. Compounds synthesized besides the compounds in Schemes 1 and 2.

observed to be time-dependent. The tricarbonyl group attached to the molecular recognition regions, namely **1** and **2**, were found to bind better than the simple tricarbonyl compound **6**. With the des-aminoacetyl **3** and **4**, the binding to Src was one order of magnitude less, and altering the tricarbonyl substitution from the 4- (**3**) to 3-position (**4**) of the phenyl ring did not change the binding significantly. This selectivity of the tricarbonyl compounds to Src SH2 prompted us to investigate the binding further by NMR. Three of the ^{13}C labeled aromatic tricarbonyl compounds (viz. **6a**–**c**) were synthesized[18] in order to study the reactions and the nature of

Table 1. Comparative binding of tricarbonyl compounds to wild-type Src SH2 and mutant Cys188Ala Src SH2

Compounds	WT Src IC_{50} (μM)	Src Cys188Ala IC_{50} (μM)	Selectivity
1	8.6	>1000	Yes
2	2.3	>1000	Yes
3	20	>1000	Yes
4	18	>1000	Yes
5	250	15% @500 μM	No
6	100	>1000	Yes
pYEEI	1.2	1.33	No

intermediate species formed. Compounds **6a–c** were subjected to NMR studies with single-labeled (^{15}N) and double-labeled (^{13}C,^{15}N) Src SH2.[19]

The ^{1}H, ^{13}C, and 2-D ^{1}H–^{15}N HSQC NMR[20] spectra were acquired initially every 1 h to check the time dependence in the binding event. The initial event was the addition of the thiol of Cys in the protein to the electrophilic carbon of **6**, which was seen by the disappearance of the peak at 91.2 ppm (^{13}C NMR is assigned to the only labeled carbon of **6a**) (Fig. 3, Table 2). Further analysis indicated that this adduct underwent a retro-Claisen rearrangement to form **16** and **17** in 24 h. Thus, the peak at 191.7 ppm, which was of a keto group attached to the phenyl ring, disappears earlier to give the carboxylic acid carbonyl at 173.0 ppm. The appearance of peaks at 83.7 ppm by ^{13}C NMR and 5.40 ppm by ^{1}H NMR indicates the presence of a secondary alcohol of **17** and also confirms the retro-Claisen rearrangement. At 70 h, the ester group was hydrolyzed to the carboxylic acid **18** and methanol. Initially we made the double-labeled (^{13}C) tricarbonyl compound **6b**, which provided almost all the information needed, but to confirm the hydrolysis of the ester at pH 7.4 as a function of time, we also synthesized the other two compounds **6a** and **6c**. A portion of the 2-D ^{1}H–^{15}N HSQC spectrum acquired within 1 h of mixing of **6a** with ^{15}N Src-SH2 is shown in blue in Figure 1a. A few cross peaks arising from the backbone HN/N of Src are shown marked with their residue number. 2-D ^{1}H–^{15}N HSQC spectrum acquired in 24 h time frame is shown in Figure 1a. The cross peaks that have moved from their positions (absence of red cross peak from the equivalent positions of blue cross peak) represent residues which are affected by the complex formation. All these residues are a part of the phosphotyrosine binding pocket of Src SH2. Noticeable among these residues are the cross peaks of Glu181 (βC1), Cys188 (βC3), Leu189 (βC4), Tyr205 (βD5), Lys206 (βD6), Ile207 (βD7), and Arg208 (βD1). The peak of Ser192 (βC7), which is not a part of any pocket is not affected at all. Moreover, cross peaks of Cys248 (βG3), and Cys241 (BG5 loop) are not affected at all (Fig. 1b). The 2-D ^{1}H–^{15}N HSQC spectrum acquired at 72 h is shown in Figure 1a in green. There are no significant differences in the spectra acquired at 24 h (red) and 72 h (green). The 2-D ^{1}H–^{15}N HSQC spectra were identical to those discussed for **6a**. The 2-D ^{1}H–^{13}C HSQC spectrum was acquired on **6c** after 30 h of mixing with double-labeled (^{13}C,^{15}N) Src SH2 and a part of the spectrum is shown in Figure 1b in red. The spectrum compared to the uncomplexed Src data is shown in green. The peaks corresponding to CysCβ-Hβ for other Cys residues of Src SH2 (at Cys241 and Cys248) are still present, indicating clearly that only Cys188 is targeted by the tricarbonyl compound **6c** (**6a** and **6b** as well). Similar decompositions of the tricarbonyl compounds were reported in the literature.[21] This disproportionation also occurred in aqueous solution in

Table 2. ^{1}H and ^{13}C NMR data for **6a–c** with double-labeled (^{13}C and ^{15}N) Src SH2 with the rearranged products **16**, **17**, and **18**

Functional group	6a	6b	6c	16	17	18
-COOMe	3.77	3.77	3.77		3.82	
-CHOH					5.40	5.10
-PhCO		191.7	191.7	173.0		
-C(OH)2		91.2	91.2			
–COOMe	168.0	168.0	168.0		169.0	

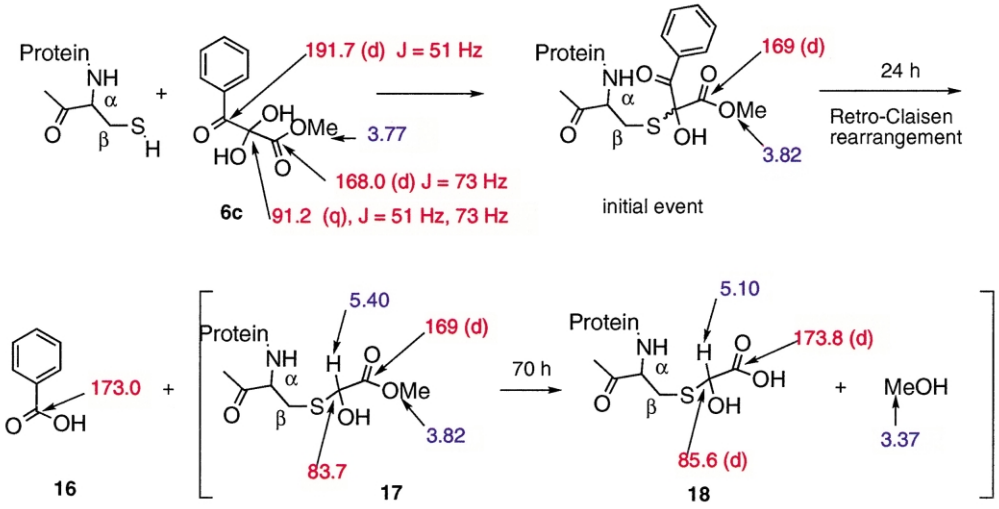


Figure 3. Sequence of events when ^{13}C labeled **6** was complexed with Src SH2 with the $^{1}H/^{13}C$ chemical shifts of each species. Blue: ^{1}H NMR chemical shifts in ppm; red: ^{13}C NMR chemical shift in ppm.

the presence of glutathione alone. Thus, it became clear that tricarbonyls would not be physiologically viable molecules. Nevertheless, in this paper we have demonstrated that the tricarbonyl compounds showed the feasibility of selectively inhibiting the Src SH2 with a reversible thiol-targeting group

Acknowledgements

The authors thank Tomi K. Sawyer for his useful suggestions in writing this manuscript. Our special thanks to Ms. Chris Stearns for her help in editing this document.

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