

Elimination of $\Delta^3 \rightarrow \Delta^2$ Isomerization in the Synthesis of a Cephem-Containing Chemical Inducer of Dimerization through a Modified Purification

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Chemical inducers of protein dimerization (CIDs) are dimeric ligands that allow proteins to be artificially dimerized *in vivo* via dimerization of the ligands' receptors.¹ First introduced in early '90s, CIDs have now been used for applications ranging from the control of gene transcription to mechanistic studies of signal transduction pathways.² It has been shown that CIDs could be used to link enzyme catalysis to reporter gene transcription *in vivo*, providing a general high-throughput assay for enzyme catalysis (Figure 1).³ This strategy hinges on using a CID with an enzyme-cleavable linker. In the proof of principle, the assay was developed around the well-studied enzyme-catalyzed reaction cephem hydrolysis by a cephalosporinase enzyme using a methotrexate-dexamethasone CID with a cleavable cephem linker (Mtx-Cephem-Dex) (Figure 1). As is commonly observed in the synthesis of cephem antibiotics, the final deprotection of the Mtx-Cephem-Dex molecule suffered from a poor yield due to $\Delta^3 \rightarrow \Delta^2$ isomerization of the cephem core.⁴ Here we report the interesting observation that this problem resulted from the purification of the final compound, rather than the deprotection *per se*. Based on this observation, we present an improved protocol for the deprotection and purification of the Mtx-Cephem-Dex CID that should have been bearing on the synthesis of cephem antibiotics generally.

Cephems have been widely used not only as antibiotics, but also as prodrugs and, most recently, as reporter molecules.⁵ Their conjugated 4,6 ring fusion system allows the cleavage of the β -lactam bond to be coupled to the expulsion of the leaving group at the C3' position. A common difficulty in the synthesis of cephem derivatives is $\Delta^3 \rightarrow \Delta^2$ isomerization of

the cephem core (Figure 2), although the Δ^3 and Δ^2 isomers can be distinguished readily by their ¹H NMR spectra⁶ (Δ^2 isomer [δ C-2 H at around 5.9 and C-4 H at around 4.7] and pure Δ^3 isomer [δ C-2 H at around 3.5]). Because Δ^2 -cephalosporins are essentially inactive,⁷ obtaining pure Δ^3 isomers is very important in this chemistry. Therefore, a number of methods have been developed to suppress this isomerization as well as to convert the Δ^2 back to the Δ^3 isomer.⁴ For example,^{4(c)} water-soluble sodium salt form of Δ^2 isomer which can be prepared by the treatment of the acid chloride with a strong tertiary amine base followed by quenching with alcohol and then smooth hydrolysis. The resulting acid can be treated with the standard method of oxidation-reduction sequence, which restored the double bond to the 3'-position through the intermediacy of the Δ^3 -sulfoxide because β,γ -unsaturated sulfoxides are thermodynamically more stable than the α,β -unsaturated sulfoxides, which results from the new stereoelectronic constraint. However, this kind of problem still remains when the molecule has some functional groups, that may be susceptible to oxidation or reduction.

Not surprisingly, an initial synthesis of the Mtx-Cephem-Dex CID yielded an impure final product in low yield.³ As shown in Scheme 1, the commercial cephem intermediate 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester (ACLE) could be readily incorporated into this synthesis. This synthesis relies on first building the Mtx and Dex halves of the molecule and then coupling them to the chemical linker. A thiol analog of Mtx was synthesized so that the final cephem substrate would have a thioether linkage at the C3' position, which acts as a leaving group when β -lactamase hydrolyzes this molecule. The *t*-butyl protecting group was cleaved with trifluoroacetic acid (TFA) to give the free terminal carboxylic acid, which is necessary for the recognition of β -lactamase. Initially, the final protected intermediate was deprotected for 1 hour in neat TFA to remove both *t*-butyl and *p*-methoxybenzyl (PMB) protecting groups.⁸ The TFA was removed imme-

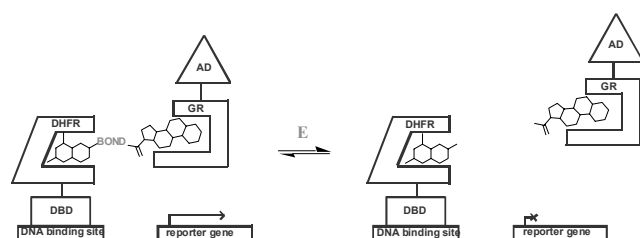


Figure 1. Complementation developed around cephem hydrolysis by cephalosporinase. A heterodimeric small molecule bridges a DNA-binding domain-receptor fusion protein and an activation domain-receptor fusion protein, activating transcription of a downstream reporter gene *in vivo*. Enzyme catalysis of either cleavage or formation of the bond between the two small molecules can be detected as a change in transcription of the reporter gene.

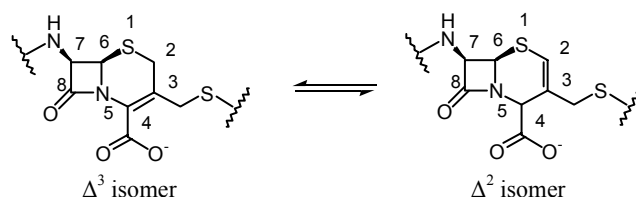


Figure 2. $\Delta^3 \rightarrow \Delta^2$ isomerization of the cephem core.

In conclusion, using the simple precipitation method we have optimized the deprotection and purification of a Dex-Cephem-Mtx CID with a cephalosporin core. Given the conventional use of cephalosporins as effective antibiotics and their new uses as useful substrates in fluorescence resonant energy transfer (FRET) and yeast three-hybrid assay to quantify gene expression, this method is expected to be generally useful to a lot of researchers using cephalosporins.

Experimental Section

Almost all of the methods for chemical synthesis were the same as reported earlier³ unless otherwise noted. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 400-MHz Fourier-transform NMR spectrometer.

The numbering of compounds reported here is exactly the same as reported.³

Synthesis of 1-4. Compounds 1-4 were synthesized as reported to give in 97, 94, 87, and 96% yields, respectively.

Synthesis of 5. Compound 4 (59.0 mg, 0.114 mmol), ACLE (45.9 mg, 0.114 mmol), and dicyclohexylcarbo-diimide (24.7 mg, 0.120 mmol) were combined and dissolved in DMF (0.15 mL). DIEA (0.0198 mL, 0.114 mmol) were then added to the solution, and the reaction was stirred at room temperature for 3 h under nitrogen. The reaction was diluted with CH₂Cl₂ (20 mL), washed with 1:1 brine/1 M NaHSO₄ (20 mL, two times). The organic phase was dried with anhydrous Na₂SO₄ and purified by silica gel column chromatography with 80:1 to 60:1 CH₂Cl₂/MeOH. A slightly yellow solid was obtained in 56% yield. *R*_f = 0.55 in 10:1 CH₂Cl₂/MeOH; ¹H NMR (400-MHz, CD₃OD); δ 7.44 (d, *J* = 10.1 Hz, 1), 7.35 (d, *J* = 8.8 Hz, 2), 6.93 (d, *J* = 8.8 Hz, 2), 6.30 (dd, *J* = 1.8, 10.1 Hz, 1), 6.10 (s, 1), 5.76 (d, *J* = 4.9 Hz, 1), 5.27 (d, *J* = 11.8 Hz, 1), 5.20 (d, *J* = 11.8 Hz, 1), 5.11 (d, *J* = 4.9 Hz, 1), 4.54 (d, *J* = 11.5 Hz, 1), 4.49 (d, *J* = 11.5 Hz, 1), 4.26 (d, *J* = 11.0 Hz, 1), 3.81 (s, 3), 3.75 (d, *J* = 18.1 Hz, 1), 3.60 (d, *J* = 18.1 Hz, 1), 3.29 (m, 1), 3.14 (m, 2), 2.75 (dt, *J* = 6.0, 13.6 Hz, 1), 2.6-2.1 (m, 6), 2.0-1.2 (m, 18), 1.1 (s, 3), 0.91 (d, *J* = 7.2 Hz, 3). ¹³C NMR (100-MHz, CD₃OD), δ 187.77, 175.58, 174.23, 169.95, 165.13, 161.60, 160.27, 154.91, 130.54, 128.70, 127.35, 126.99, 125.75, 124.04, 113.90, 102.57, 100.83, 87.22, 72.33, 71.96, 68.11, 59.81, 58.42, 54.91, 49.63, 49.41, 44.06, 43.31, 30.58, 36.16, 35.60, 35.53, 35.09, 34.90, 32.65, 31.51, 29.98, 29.27, 28.08, 27.10, 25.98, 22.95, 17.10, 14.49; MS *m/z* 534.4 (MH⁺); HRMS, *m/z* 534.3211 (MH⁺), calculated 534.3231; IR 3416 (br), 3044, 2930, 2860, 1785, 1726, 1360, 1627, 1517, 1450, 1389, 1361, 1300, 1244, 1170, 1099, 1065, 1030, 1020, 982, 950, 928, 889, 820.

Synthesis of 6 and 7. Compound 6 and 7 were synthesized as reported³ to give in 57% and 93% yield, respectively.

Synthesis of 8. Compound 7 (394 mg, 0.50 mmol) and the hydrobromide salt of 2,4-diamino-6-bromomethyl-pteridine (435 g, 1.1 mmol) were dissolved in DMA (1.25 mL) and stirred in a 55 °C oil bath overnight. The reaction was diluted with CH₂Cl₂ and the product was purified by silica gel column chromatography with 10:1 and 5:1 CH₂Cl₂/MeOH in 97% yield. ¹H NMR (400-MHz, CD₃OD) δ 8.65 (s, 2), 7.76 (d, *J* =

9.0 Hz, 4), 6.85 (d, *J* = 9.0 Hz, 4), 4.93 (s, 4), 4.45 (dd, *J* = 4.5 Hz, 2), 3.41 (t, *J* = 6.6 Hz, 4), 3.29 (s, 6), 2.72 (t, *J* = 6.7 Hz, 4), 2.38 (t, *J* = 6.7 Hz, 4), 2.21 (m, 2), 2.10 (m, 2), 1.47 (s, 18); ¹³C NMR (100-MHz, CD₃OD) δ 176.1, 173.8, 171.0, 165.5, 165.1, 156.6, 154.0, 151.1, 150.0, 131.2, 124.3, 123.1, 113.5, 83.8, 57.5, 55.6, 40.7, 40.6, 39.3, 34.2, 29.2, 28.9.

Synthesis of 9. The disulfide compound 8 (440 mg, 0.387 mmol) was dissolved in DMF (1.9 mL) and water (0.2 mL). After being purged with N₂, tributylphosphine (0.107 mL, 0.426 mmol) was added, and the reaction was stirred at room temperature overnight. The product was purified by silica gel column chromatography with 40:1 and 10:1 CH₂Cl₂/MeOH in 99% yield. ¹H NMR (400-MHz, CD₃OD) δ 8.61 (s, 1), 7.78 (d, *J* = 9.8 Hz, 2), 6.89 (d, *J* = 9.7 Hz, 2), 4.89 (s, 2), 4.47 (m, 1), 3.31 (m, 2), 3.28 (s, 3), 2.55 (t, *J* = 6.9 Hz, 2), 2.38 (m, 2), 2.23 (m, 1), 2.09 (m, 1), 1.49 (s, 9); ¹³C NMR (100-MHz, CD₃OD) δ 174.19, 171.55, 168.76, 163.19, 161.07, 151.91, 149.21, 148.79, 129.38, 122.51, 121.01, 111.73, 82.45, 55.91, 53.80, 43.23, 39.45, 32.67, 27.93, 23.93; MS, *m/z* 570.3 (MH⁺); HRMS, *m/z* 570.2590 (MH⁺) calculated 570.2611; IR 3335 (br), 2974, 2928, 2350, 1726, 1635, 1606, 1554, 1511, 1450, 1365, 1135, 1344, 1205, 1151, 1104, 919, 831, 762.

Synthesis of 10. Compound 10 was synthesized as reported³ in 75% yield.

Synthesis of 11. To compound 10 (17.9 mg, 0.0128 mmol) was added thioanisole (1.65 μL, 0.141 mmol) followed by TFA (0.15 mL). The reaction was stirred at room temperature for 15 min under nitrogen. Then the reaction mixture was briefly concentrated in vacuo and the residue was suspended in 50 mL of CH₂Cl₂. And after a 10 sec sonication, the mixture was delivered to 50 mL Falcon tube, which was then centrifuged with 2000 rpm for 5 min. After the removal of the supernatant, the dilution and centrifuge step were repeated. This procedure gave the yellowish solid of desired product in 90% yield. ¹H NMR (400-MHz, 4:1 CD₃OD /CDCl₃) δ 8.65 (s, 1), 7.78 (d, *J* = 8.9 Hz, 2), 7.41 (d, *J* = 10.1 Hz, 1), 6.84 (d, *J* = 8.8 Hz, 2), 6.29 (dd, *J* = 1.8, 10.1 Hz, 1), 6.09 (s, 1), 5.66 (d, *J* = 4.6 Hz, 1), 5.07 (d, *J* = 5 Hz, 1), 4.91 (s, 2), 4.58 (m, 1), 4.25 (d, *J* = 9.3 Hz, 1), 3.94 (d, *J* = 15.3 Hz, 1), 3.70 (q, *J* = 17.9 Hz, 2), 3.60 (d, *J* = 15.3 Hz, 1), 3.37 (m, 3), 3.22 (s, 3), 3.14 (m, 3), 2.70 (m, 1), 2.64 (t, *J* = 6.8 Hz, 2), 2.5-2.0 (m, 10), 1.90 (m, 1), 1.75 (q, *J* = 11.8 Hz, 1), 1.69-1.17 (m, 16), 1.10 (s, 3), 0.90 (d, *J* = 7.2 Hz, 3); ¹³C NMR (75 MHz, 4:1 CD₃OD /CDCl₃) δ 188.20, 175.89, 174.20, 169.87, 165.27, 163.92, 163.62, 156.64, 155.02, 152.67, 151.63, 149.35, 145.76, 131.36, 129.13, 126.85, 125.25, 124.24, 122.19, 121.71, 111.73, 102.59, 100.89, 87.28, 72.30, 71.93, 59.71, 58.88, 56.98, 53.19, 49.66, 44.09, 39.83, 39.70, 39.36, 36.19, 35.83, 35.59, 35.10, 34.92, 33.36, 32.88, 32.75, 31.67, 30.69, 29.97, 29.38, 28.08, 27.86, 27.67, 27.13, 26.01, 23.15, 17.29, 14.71; MS, *m/z* 1403.9 (MH⁺), 1227.50 (MH⁺).

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