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

Sang Soo Hah* **and Jungahn Kim**

Department of Chemistry and Research Institute for Basic Sciences, Kyung Hee University, Seoul 130-701, Korea
**E-mail: sshah@khu.ac.kr*
Received January 6, 2009, Accepted January 28, 2009

Key Words: Cephem, $\Delta^3 \rightarrow \Delta^2$ Isomerization, Chemical inducer of protein dimerization

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 ^{1}H NMR spectra[6] (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 2H 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,[4c,d] 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.

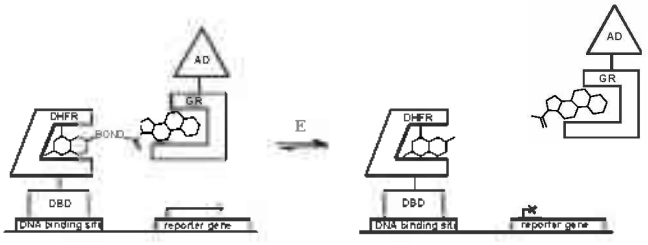


Figure 1. Complementation developed around cephem hydrolysis by cephaloseporinase. A heterodimeric small molecule bridges a DNA-binding domain-receptor fusion protein and an activation domain-receptor fusion protein, activating transcription of a downstream reporter gene *in vivo*. Enzyme catalysis of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

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-

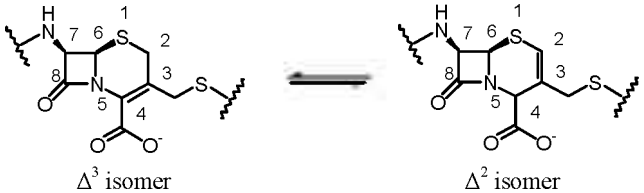
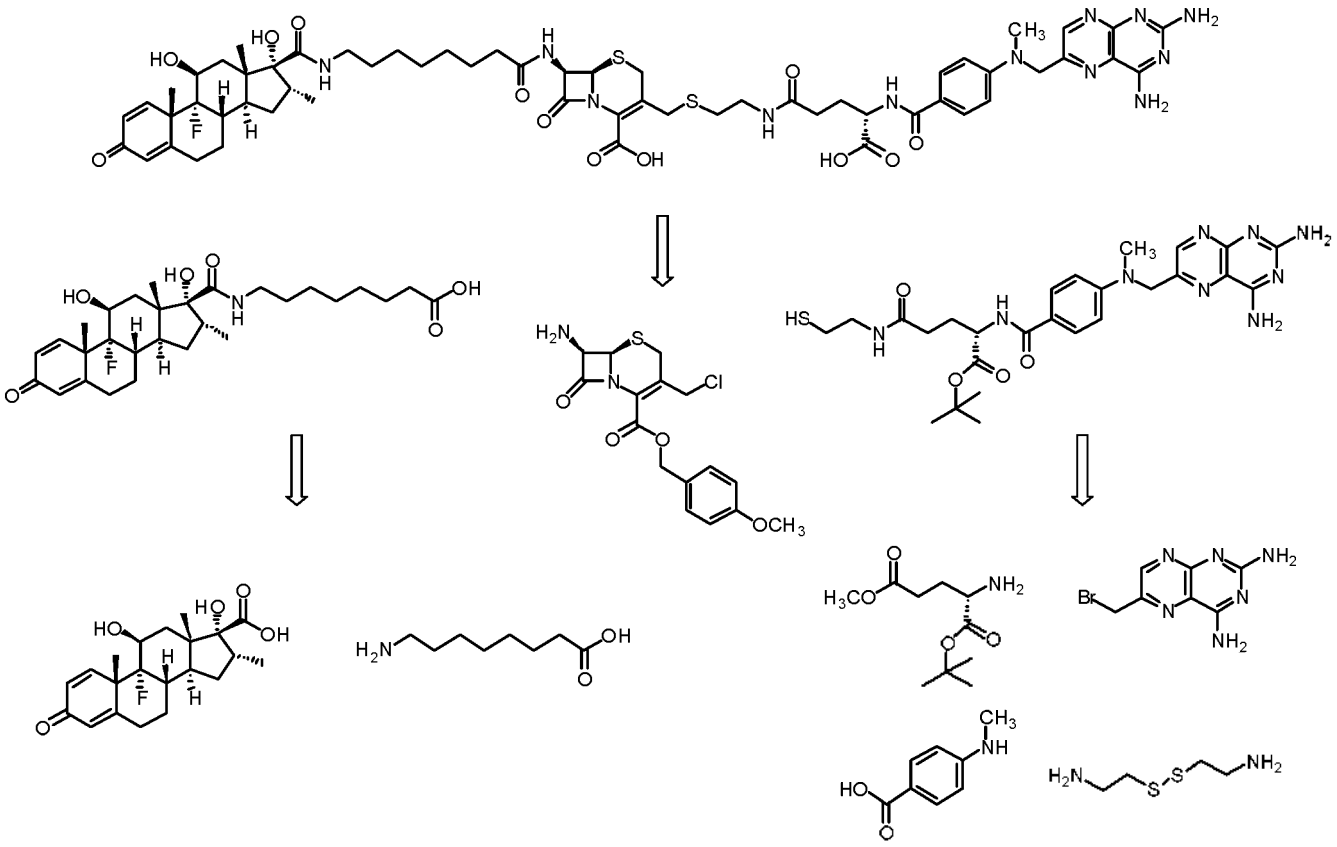


Figure 2. $\Delta^3 \rightarrow \Delta^2$ isomerization of the cephem core.



Scheme 1. Retrosynthetic analysis of Mtx-Cephem-Dex.

diately by azotroping with toluene. The product was purified either by silica gel chromatography using a gradient of methanol in methylene chloride or by HPLC using a gradient of acetonitrile and water. Both methods yielded a mixture of products that could not be further separated with only around 10% yield, so the impurities could not be determined unambiguously. Inspection of the ^{1}H NMR and mass spectra suggested the major impurity was the $\Delta^3 \rightarrow \Delta^2$ isomer of the final product. In addition, the mixture isolated by silica gel chromatography gave not only the desired M^- peak, but also a M+32 peak in mass spectra. This impurity is assumed to be Michael addition of MeOH across the olefin in the cephem core. The only other reasonable site for the Michael adduct is the A ring of Dex. The ^{1}H NMR, however, shows the A ring to be intact.

Based on these observations, it was reasoned that milder purification conditions could suppress both $\Delta^3 \rightarrow \Delta^2$ isomerization and formation of the Michael adduct. Because it was already reported that although the Δ^3 isomer can readily equilibrate under basic conditions with the Δ^2 isomers, the free acids can also equilibrate at a relatively lower rate,[5(a)] and a shortened reaction time was tried compared with that of the literature.[8] Interestingly, neither the $\Delta^3 \rightarrow \Delta^2$ isomerization nor the Michael adduct was not detected in the crude reaction mixture prior to purification. Thus, protocol was developed for purification of final product.[9] It was found that the desired product can be isolated with 90% yield, after a short (15 min) TFA/thioanisole treatment and removal of most TFA, by adding methylene chloride until the product precipitated out from the solution. More importantly, the product thus obtained gave a beautiful ^{1}H NMR and MS spectra.[9] ^{13}C, COSY, HSQC NMR experiments have also been carried out and they all support the correct structure.

To further support that the impurity resulted from purification, rather than chemical reaction, the pure product obtained using the precipitation method was subjected to the initial conditions for silica gel purification. The solubility changed dramatically after the silica gel column. And on the NMR spectrum, the peaks from the cephem portion were no longer discernable and the major peak in MS is the M+32 peak. Therefore, it can be concluded that either the β-lactam is opened by the attack of nucleophilic solvent such as methanol or water, or the nucleophilic solvent added to the double bond *via* a Michael addition reaction, catalyzed by silica gel which is a weak Lewis acid. Similarly, it could be assumed that the Dex-Cephem-Mtx compound is not stable under the HPLC condition used, where decomposition and isomerization could have happened.

As expected, the Mtx-Cephem-Dex molecule obtained by the method mentioned above was active both *in vitro* and *in vivo*.[10(9)] It is shown to be a good substrate for the cephalosporinase enzyme by monitoring the cleavage of the β-lactam bond at 412 nm. The Mtx-Cephem-Dex compound is also a good CID in the yeast 3-hybrid system as evidenced by the liquid *o*-nitrophenyl β-D-galactopyranoside (ONPG) assay, where yeast strains expressing P99 cephalosporinase were grown in liquid culture and assayed for β-galactosidase activity with ONPG as a substrate (data not shown).

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 aynthesis were the same as reported earlier[3] 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 CH_2Cl_2 (20 mL), washed with 1:1 brine/1 M $NaHSO_4$ (20 mL, two times). The organic phase was dried with anhydrous Na_2SO_4 and purified by silica gel column chromatography with 80:1 to 60:1 CH_2Cl_2/MeOH. A slightly yellow solid was obtained in 56% yield. R_f = 0.55 in 10:1 CH_2Cl_2/MeOH: ^{1}H NMR (400-MHz, CD_3OD): 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). ^{13}C NMR (100-MHz, CD_3OD), 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 reported[3] 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 CH_2Cl_2 and the product was purified by silica gel column chromatography with 10:1 and 5:1 CH_2Cl_2/MeOH in 97% yield. ^{1}H NMR (400-MHz, CD_3OD) 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); ^{13}C NMR (100-MHz, CD_3OD) 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 N_2, 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 CH_2Cl_2/MeOH in 99% yield. ^{1}H NMR (400-MHz, CD_3OD) 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); ^{13}C NMR (100-MHz, CD_3OD) 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 reported[3] 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 CH_2Cl_2. 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. ^{1}H NMR (400-MHz, 4:1 CD_3OD /$CDCl_3$) 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, 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); ^{13}C NMR (75 MHz, 4:1 CD_3OD /$CDCl_3$) 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+).

Acknowledgments. We thank Drs. Virginia Cornish and Hening Lin for their valuable guidance and help. This work was supported by the Kyung Hee University Research Fund in 2008 (KHU-20081559).

References

1. (a) Spencer, D. M.; Wandless, T. J.; Schreiber, S. L.; Crabtree, G. R. *Science* **1993**, *262*,1019-1024; (b) Licitra, E. J.; Liu, J. O. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 12817-12821; (c) Griffith, E. C.; Licitra, E. J.; Liu, J. O. *Methods Enzymol.* **2000**, *328*, 89; (d) Lin, H.; Cornish, V. *Angew. Chem. Int. Ed.* **2002**, *41*, 4402-4425.
2. (a) Farrar, M. A.; Alberolalla, J.; Permutter, R. M. *Nature* **1996**, *383*, 178-181; (b) Belshaw, P.; Ho, S.; Crabtree, G.; Schreiber, S. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 4604-4607; (c) Diver, S.; Schreiber, S. *J. Am. Chem. Soc.* **1997**, *119*, 5106-5109; (d) Amara, J.; Clackson, T.; Rivera, V. M.; Guo, T.; Keenan, T.; Natesan, S.; Pollock, R.; Yang, W.; Courage, N. L.; Holt, D. A.; Gilman, M. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 10618-10623; (e) Clarkson, T.; Yang, W.; Rozamus, L.; Hetada, M.; Amara, J.; Rollins, C.; Stevenson, L.; Magari, S.; Wood, S.; Courage, N. L.; Lu, X.; Cerasoli Jr., F.; Gilman, M.; Holt, D. A. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 10437-10442.
3. Baker, K.; Bleczinski, C.; Lin, H.; Salazar-Jimenez, G.; Sengupta, D.; Krane, S.; Cornish, V. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 16537-16542.
4. (a) Kukolja, S. *J. Med. Chem.* **1970**, *13*, 1114-1117; (b) Murphy, C.; Koehler, R. *J. Org. Chem.* **1970**, *35*, 2429-2430; (c) Kaiser, G.; Cooper, R.; Koehler, R.; Murphy, C.; Webber, J.; Wright, J.; Van Heyningen, E. *J. Org. Chem.* **1970**, *35*, 2430-2433; (d) Frere, J.-M.; Kelly, J. A.; Klein, D.; Ghuysen, J.-M.; Claes, P.; Vanderhaeghe, H. *Biochem. J.* **1982**, *203*, 223; (e) Farina, V.; Baker, S.; Hauck, S. *J. Org. Chem.* **1989**, *54*, 4962-4966; (f) Albrecht, H.; Beskid, G.; Chan, K.-K.; Christenson, J.; Cleeland, R.; Deitcher, K.; Georgopapadakou, N.; Keith, D.; Pruess, D.; Sepinwall, J.; Specian Jr., A.; Then, R.; Weigele, M.; West, K.; Yang, R. *J. Med. Chem.* **1990**, *33*, 77-86; (g) Buynak, J.; Doppalapudi, V.; Adam, G. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 853-857.
5. (a) Sammes, P. *Chem. Rev.* **1976**, *76*, 113-155; (b) Cooper, R.; Hartfield, L.; Spry, D. *Acc. Chem. Res.* **1973**, *6*, 32-40; (c) Durckheimer, W.; Adam, F.; Fischer, G.; Kirrstetter, R. *Adv. Drug Res.* **1988**, *17*, 61; (d) Zlokarnik, G.; Negulescu, P. A.; Knapp, T. E.; Mere, L.; Burres, N.; Feng, L.; Whitney, M.; Roemer, K.; Tsien, R. Y. *Science* **1998**, *279*, 84-88.
6. (a) Murphy, C.; Koehler, R. *J. Org. Chem.* **1970**, *35*, 2429-2430; (b) Balsamo, A.; Crotti, P.; Macchia, B.; Macchia, F.; Nannini, G.; Dradi, E.; Forgione, A. *J. Org. Chem.* **1976**, *41*, 2150-2153.
7. Webber, J.; Van Heyningen, E.; Vasileff, R. *J. Am. Chem. Soc.* **1969**, *91*, 5674-5675.
8. Torii, S.; Hanaka, H.; Taniguchi, M.; Kameyama, Y. *J. Org. Chem.* **1991**, *56*, 3633-3637.
9. Sengupta, D.; Lin, H.; Goldberg, S.; Mahal, J. J.; Cornish, V. *Biochemistry* **2004**, *43*, 3570-3581.