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A Practical Synthesis of Nitrocefin

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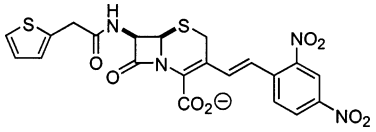
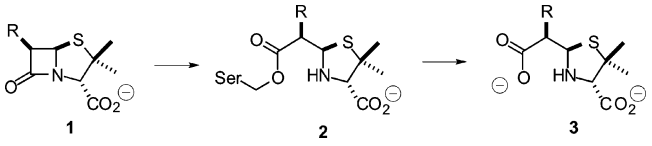


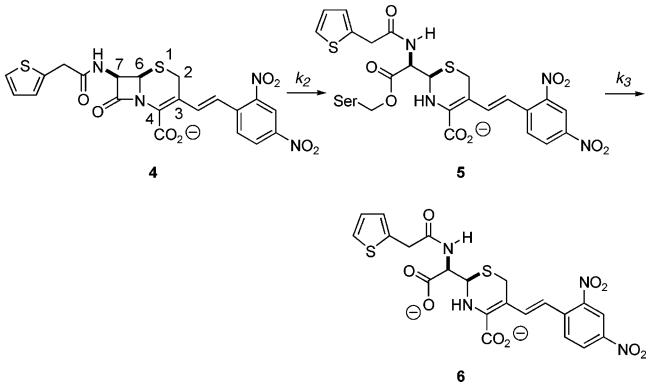
Nitrocefin is a key reagent for high and low throughput assays of the activities of penicillin-binding proteins (PBPs) and β-lactamases, the former used for discovery of antibiotics and the latter for inhibitors of resistance determinants for β-lactam antibiotics. This compound is commercially available but is prohibitively expensive because of the circuitous routes to its synthesis. We describe herein a three-step synthesis of nitrocefin that gives an overall yield of 44%. This is a practical route to the synthesis of this key reagent for drug discovery.

β-Lactam antibiotics (penicillins, caphalosporins, etc.) acylate the active site serines of penicillin-binding proteins (PBPs; **1** → **2**, as depicted for a penicillin), a reaction that deprives bacteria of their physiological function and kills them. β-Lactamases are related bacterial enzymes that have acquired the ability to hydrolyze the acyl-enzyme species (**1** → **2** → **3**), whereby they turn over these antibiotics, resulting in resistance to them. Inhibitors of both PBPs and β-lactamases are highly sought, as one results in antibiotic activity and the other the means to overcoming resistance.[1]



1 **2** **3**

A large-scale search for inhibitors of both families of enzymes requires rapid, sensitive, and chromogenic assays. Fortunately, nitrocefin (**4**), a cephalosporin, meets these requirements as an assay reagent. Nitrocefin acylates the active-site serine of these enzymes, a reaction that results in species **5** for the PBP reaction, and after hydrolysis of the acyl-enzyme species, in compound **6** for the β-lactamase reaction. The opening of the β-lactam bond in nitrocefin results in both a color change (yellow to deep red) and a large change in $\Delta\epsilon_{485nm}$ of $+15\,600\ cm^{-1}\ M^{-1}$.[2] These characteristics are suitable for convenient detection of both PBP and β-lactamase activities. For PBPs, detection at a near micromolar level of protein can be made. Because of turnover of nitrocefin by β-lactamases, activity detection at the low nanomolar level is readily possible for these enzymes.



4 **5** **6**

Nitrocefin is commercially available but is prohibitively expensive (currently $7,600 per gram). The cost in part reflects the high demand for this cephalosporin but also the fact that the existing syntheses for this useful reagent are multistep and low-yielding processes. Two syntheses of nitrocefin have been described in the literature, one of which has appeared only in the patent literature.[3] The patent chemistry reported by Glaxo utilizes a semisynthetic C_3-methyl cephalosporin in radical chemistry to introduce a halide to the position (cephem numbering is given on the structure of **4**), which was used in Wittig chemistry to install the dinitrostyryl moiety. They needed to oxidize the sulfur at position 1, as cephem sulfoxides are known not to undergo the problematic $\Delta^3 \rightarrow \Delta^2$ isomerization of the dihydrothiazine ring (discussed below). The cephalosporin oxide had to be reduced at the end of this synthesis.

The second report also follows the same type of strategy (oxidation of the cephem sulfur and the requisite reduction at the end of the synthesis).[4] However, they had to incorporate an additional enzymic step for the removal of the C_7-acyl moiety and the subsequent acylation by the 2-thienylacetyl group present in the structure of nitrocefin. Both synthetic strategies are multistep and low-yielding.

We have developed a three-step high-yielding synthesis of nitrocefin from a commercially available reagent, 7β-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (**7**; Scheme 1). As mentioned earlier, a salient difficulty in syntheses of cephalosporins is that when the C_4 carboxylate is protected (as with the *p*-methoxybenzyl ester in the case of **7**), the cephalosporin becomes prone to an undesirable $\Delta^3 \rightarrow \Delta^2$ isomerization of the dihydrothiazine ring in the presence of even weak bases such as carboxylates or

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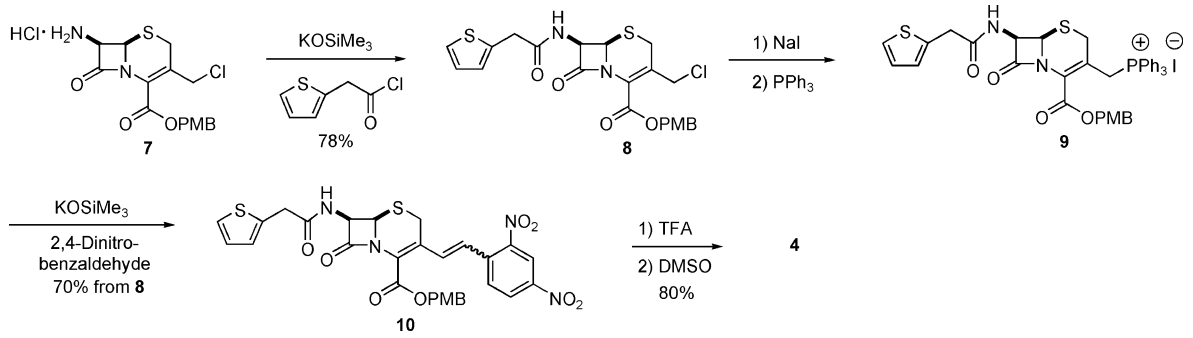
SCHEME 1



pyridines.[5] We have discovered that this undesirable isomerization is not seen by the use of potassium trimethylsilanolate as base, such as shown in two steps of the synthesis in Scheme 1. This basic reagent is soluble in organic solvents, which obviates the need for use of aqueous or mixed aqueous/organic conditions.

As shown in Scheme 1, the 7-amino group of cephalosporin **7** was acylated with 2-thienylacetyl chloride in the presence of stoichiometric potassium trimethylsilanolate to give compound **8**. The chloro moiety of **8** was converted to iodo by the Finkelstein reaction, which was allowed to undergo reaction with triphenylphosphine in situ to result in **9**. The Wittig reaction of compound **9** with 2,4-dinitrobenzaldehyde was also carried out in the presence of potassium trimethylsilanolate ($KOSiMe_3$), a reaction that proceeded in 70% yield (from compound **8**) to afford a 7:1 mixture of *Z*:*E* isomers (**10**). Potassium trimethylsilanolate has been used previously for mild hydrolyses of carboxylic esters[6] or nitrile derivatives.[7] There is no report of the use of this reagent for Wittig reaction in the literature that we are aware of. This reagent is useful as a good base for the Wittig reaction in anhydrous conditions. The yield was as good as typical Wittig reactions with cephalosporin derivatives (aqueous NaOH or $NaHCO_3$ in methylene chloride).[3,4,8−10] Deprotection of the *p*-methoxybenzyl group of compound **10** was achieved by treatment with trifluoroacetic acid at ice−water temperature for 15 min, resulting in nitrocefin as a mixture of *Z* and *E* isomers (the ratio became 6:5). The *Z* to *E* isomerization for some cephalosporin derivatives has been reported in the literature during the acid-promoted deprotection of 4-carboxylates with TFA[8] or with Lewis acid ($TiCl_4$, $SnCl_4$, etc)[4,11] A more prolonged treatment with TFA resulted in an increased ratio of *E*/*Z* but was accompanied by the decomposition of the desired product. However, we found that storage of the mixture of the isomers of **4** in 10% DMSO in chloroform over 24 h resulted in a clean conversion of the *Z* to *E* isomer. A similar isomerization has been reported in the literature under this condition.[9] Figure 1 documents the progress of isomerization in an NMR tube.

The important aspects of this synthetic route to nitorcefin are the entire avoidance of the well-precedented and undesirable $\Delta^3 \rightarrow \Delta^2$ isomerization of the dihydrothiazine ring and the facility of the complete conversion of the *Z* to *E* isomer. We hasten to add that chromatographic separation of the Δ^3 and Δ^2 isomers is difficult and often the need for oxidation/reduction at the cephem sulfur is mandated. However, the synthetic route presented in Scheme 1 bypasses these steps in simplifying the procedures considerably. The three-pot synthesis of nitrocefin was accomplished in 44% overall yield.

Experimental Section

***p*-Methoxybenzyl (6*R*,7*R*)-3-Chloromethyl-7β-(2-thienylacetamido)-3-cephem-4-carboxylate (8).** Potassium trimethylsilanolate (2.85 g, 20.0 mmol, 90% purity) in CH_3CN (30 mL) and 2-thienylacetyl chloride (1.30 mL, 10.0 mmol) were added simultaneously to a suspension of compound **7** (4.00 g, 10.0 mmol) in CH_2Cl_2 (50 mL) over 1 h in an ice−water bath. The resulting suspension was stirred at room temperature for 1 h, and the solvent was then evaporated under reduced pressure. The residue was taken up with CH_2Cl_2 and water, and the layers were separated. The organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and evaporated. The resulting solid material was recrystallized from ethyl acetate and hexane to afford the desired product (3.80 g, 78%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.41, 3.60 (2d, 2H, J = 18.2 Hz, H_2), 3.79 (s, 3H, OCH_3), 3.83 (s, 2H, thiophene-CH_2), 4.39, 4.52 (2d, 2H, J = 11.9 Hz, CH_2Cl), 4.92 (d, 1H, J = 5.1 Hz, H_6), 5.19 (s, 2H, CH_2Ar), 5.81 (dd, 1H, J = 5.1 Hz, J = 9.1 Hz, H_7), 6.65 (d, 1H, J = 9.1 Hz, NH), 6.88 (d, 2H, J = 8.6 Hz, ArH), 6.95 (m, 1H, thiophene-H), 6.98 (dd, 1H, J = 3.5 Hz, J = 5.1 Hz, thiophene-H), 7.24 (dd, 1H, J = 1.5 Hz, J = 5.1 Hz, thiophene-H), 7.32 (d, 2H, J = 8.6 Hz, ArH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 27.3 (C_2), 37.2 (thiophene-CH_2), 43.4 (CH_2Cl), 55.4 (OCH_3), 57.8 (C_6), 59.3 (C_7), 68.4 (CH_2Ar), 114.1, 125.6, 126.1, 126.5, 126.7, 127.6, 127.9, 130.9, 134.9, 160.1, 161.2, 164.8, 170.4; MS (ESI) *m/z* 515.07 $[M + Na]^+$.

***p*-Methoxybenzyl (6*R*,7*R*)-3-(2,4-Dinitrostyryl)-7β-(2-thienylacetamido)-3-cephem-4-carboxylate (10).** A mixture of compound **8** (1.00 g, 2.0 mmol), sodium iodide (1.52 g, 10.1 mmol), and triphenylphosphine (0.96 g, 3.7 mmol) in methylethyl ketone (15 mL) was stirred overnight in the dark at room temperature. The resulting suspension was filtered through a small layer of silica gel and washed with CH_2Cl_2 and acetone. The combined filtrate was evaporated under reduced pressure and was taken up into CH_2Cl_2 (20 mL). Potassium trimethylsilanolate (0.27 g, 1.9 mmol, 90% purity) in CH_3CN (5 mL) was added to the above solution at −10 °C in the dark. After 1 h, 2,4-dinitrobenzaldehyde (0.39 g, 2.0 mmol) in CH_2Cl_2 (10 mL)

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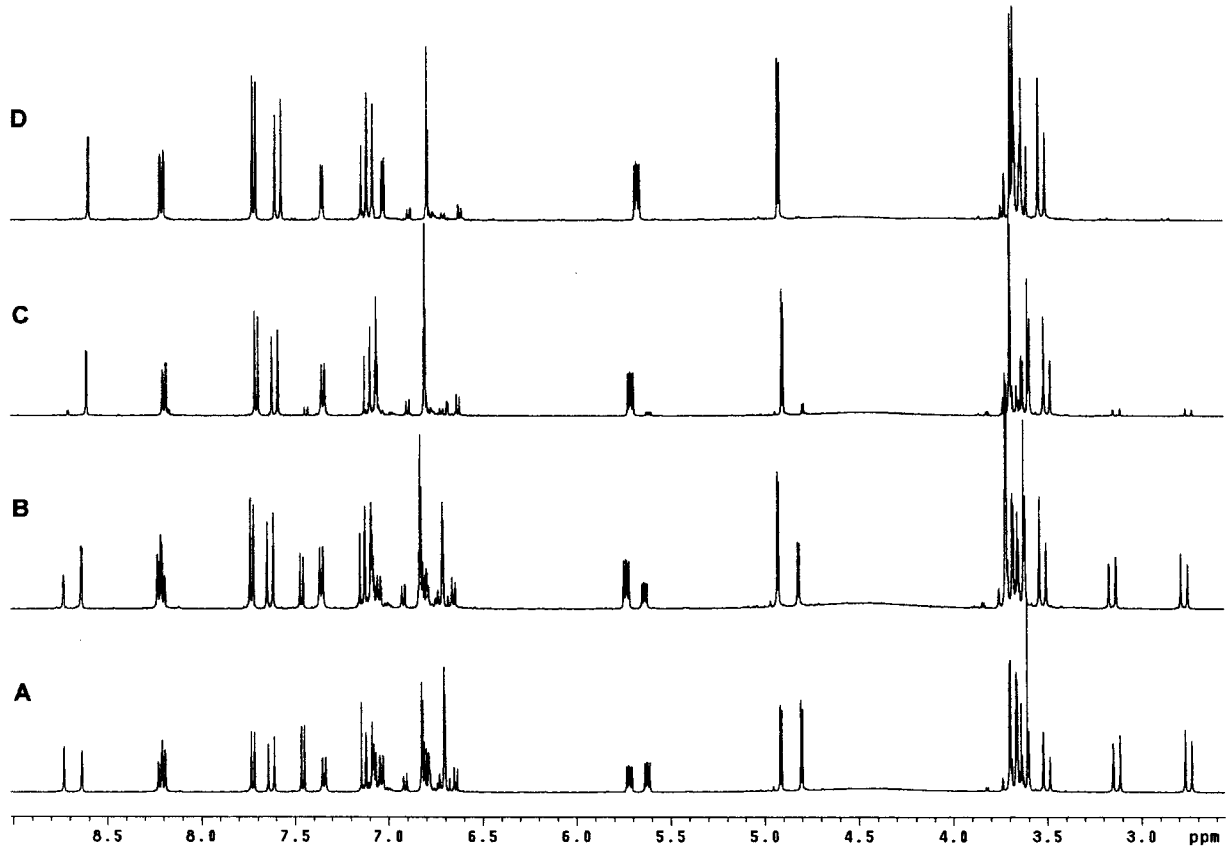

FIGURE 1. Time-course of *Z* to *E* isomerization (a 6:5 mixture at the beginning) in 10% DMSO-d_6 in chloroform-*d* in a sealed NMR tube at room temperature at (A) 0, (B) 6, (C) 12, and (D) 24 h.

was added at −10 °C. The solution was brought to room temperature after 2 h, and the mixture was stirred for 3 h. The suspension was filtered through a small layer of silica gel and was washed with CH_2Cl_2. The combined filtrate was evaporated under reduced pressure, and the residue was taken up into CH_2Cl_2 and was washed with water and brine. The organic layer was then dried over $MgSO_4$, filtered, and evaporated. The crude product was purified by column chromatography on silica gel (2:1 to 1.5:1, hexane/ethyl acetate) to give the title compound (0.90 g, 70%) as a 7:1 mixture of *Z* and *E* isomers: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.92, 3.31 (2d, 2H, J = 18.3 Hz, H_2), 3.80 (s, 3H, OCH_3), 3.82−3.83 (m, 2H, thiophene-CH_2), 4.93 (d, 1H, J = 4.9 Hz, H_6), 5.15 (s, 2H, CH_2Ar), 5.82 (dd, 1H, J = 4.9 Hz, J = 8.9 Hz, H_7), 6.58 (d, 1H, J = 9.4 Hz, NH), 6.80 (d, 1H, J = 11.9 Hz, C=CH), 6.87 (d, 2H, J = 9.9 Hz, ArH), 6.95 (m, 1H, thiophene-H), 7.00 (dd, 1H, J = 3.5 Hz, J = 4.9 Hz, thiophene-H), 7.26 (dd, 1H, J = 1.2 Hz, J = 5.2 Hz, thiophene-H), 7.31 (d, 2H, J = 9.4 Hz, ArH), 7.55 (d, 1H, J = 8.9 Hz, ArH), 8.31 (dd, 1H, J = 2.5 Hz, J = 8.4 Hz, ArH), 8.87 (d, 1H, J = 2.5 Hz, ArH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 28.6 (C_2), 37.1 (thiophene-CH_2), 55.4 (OCH_3), 58.5 (C_6), 59.5 (C_7), 68.3 (CH_2Ar), 114.1, 120.7, 125.8, 126.2, 126.6, 126.7, 127.0, 127.6, 128.0, 130.8, 132.0, 132.9, 134.9, 138.5, 147.4, 147.2, 160.1, 161.6, 164.7, 170.2; MS (ESI) *m/z* 659.10 $[M + Na]^+$.

(6*R*,7*R*)-3-(2,4-Dinitrostyryl)-7β-(2-thienylacetamido)-3-cephem-4-carboxylic Acid (4). Compound **10** (0.20 g, 0.3 mmol) in anhydrous CH_2Cl_2 (2 mL) was treated in the dark with trifluoroacetic acid (1 mL) and anisole (0.2 mL) for 15 min at ice−water temperature. The volatiles were removed rapidly under reduced pressure. The residue was taken up into benzene and was re-evaporated. The residue was triturated with diethyl ether, resulting in a light yellow precipitate. The precipitate was filtered and washed with diethyl ether. The yellow residue was dissolved in 6 mL of 10% DMSO in chloroform and was stirred at room temperature for 24 h. The solution was evaporated under reduced pressure, and the residue was triturated with diethyl ether. The resulting precipitate was filtered and recrystallized from methanol to yield the title compound as the single *E* isomer (120 mg, 80%). The NMR spectra were identical to the reported values and to those of the authentic commercial samples.[4] The *E* and *Z* mixture of **4** before isomerization: ^{1}H NMR (500 MHz, 10% DMSO-d_6 in $CDCl_3$) δ 2.75, 3.13 (2d, 2H, J = 18.3 Hz, *Z*-H_2), 3.50 (1d, 1H, J = 17.3 Hz, *E*-H_2), 3.60−3.69 (m, 5H, *E* and *Z*-thiophene-CH_2 and H_2), 4.80 (d, 1H, J = 4.5 Hz, *Z*-H_6), 4.91 (d, 1H, J = 4.9 Hz, *E*-H_6), 5.62 (dd, 1H, J = 4.9 Hz, J = 8.9 Hz, *Z*-H_7), 5.72 (dd, 1H, J = 4.9 Hz, J = 8.9 Hz, *E*-H_7), 6.70−7.14 (m, 8H, *E* and *Z*-thiophene-H, C=CH), 7.35 (d, 1H, J = 8.4 Hz, *E*-NH), 7.46 (d, 1H, J = 8.4 Hz, *Z*-NH), 7.60 (d, 1H, J = 16.3 Hz, *E*-C=CH), 7.72 (d, 1H, J = 8.4 Hz, *E*-ArH), 8.19−8.23 (m, 2H, ArH), 8.64 (d, 1H, J = 2.5 Hz, *E*-ArH), 8.73 (d, 1H, J = 2.0 Hz, *Z*-ArH). Compound **4** in pure *E* form: mp 167−169 °C (decomp); ^{1}H NMR (500 MHz, 5% DMSO-d_6 in $CDCl_3$) δ 3.50, 3.63 (2d, 2H, J = 17.6 Hz, H_2), 3.66 (dd, 2H, J = 15.8 Hz, J = 24.2 Hz, thiophene-CH_2), 4.91 (d, 1H, J = 4.9 Hz, H_6), 5.67 (dd, 1H, J = 4.9 Hz, J = 8.4 Hz, H_7), 6.75−6.80 (m, 2H, thiophene-H), 7.03 (d, 1H, J = 5.4 Hz, thiophene-H), 7.11 (d, 1H, J = 16.0 Hz, C=CH), 7.60 (d, 1H, J = 16.0 Hz, C=CH), 7.73 (d, 1H, J = 8.9 Hz, ArH), 8.22 (dd, 1H, J = 2.2 Hz, J = 8.7 Hz, ArH), 8.40 (d, 1H, J = 8.9 Hz, NH), 8.62 (d, 1H, J = 2.0 Hz, ArH).

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Supporting Information Available: NMR spectra for the synthetic molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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