Synthesis of the β-Lactamase Indicators Cefesone and Nitrocefin

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Abstract: The synthesis of the β-lactamase indicators Cefesone [(6*R*,7*R*)-3-(2,4-dinitrostyryl)-7-(phenylacetamido)ceph-3-em-4-carboxylic acid] and Nitrocefin [(6*R*,7*R*)-3-(2,4-dinitrostyryl)-7-(2-thienylacetamido)ceph-3-em-4-carboxylic acid] from *tert*-butyl (1*S*,6*R*,7*R*)-3-bromomethyl-1-oxo-7-(phenylacetamido)ceph-3-em-4-carboxylate is reported. Phosphonylation of the latter compound with triphenylphosphine gave the corresponding phosphonium derivative in 93% yield. Reduction followed by Wittig coupling with 2,4-dinitrobenzaldehyde gave a 1:12 mixture of *E*- and *Z*-isomers in 83% yield. The *tert*-butyl protecting group was removed with titanium tetrachloride to give Cefesone as the pure crystalline *E*-isomer in 63% yield. De-acylation was achieved by enzymatic hydrolysis in 77% yield. Finally the 2-thienylacetyl side chain was introduced to give crystalline Nitrocefin in 67% yield.

Key words: Cefesone, cephalosporin, Nitrocefin, β-lactamase indicator, penicillin acylase

Cefesone (**4**) and Nitrocefin (**6**) are chromogenic cephalosporins useful in the determination of.β-lactamase activity in biological samples. Upon enzymatic hydrolysis of the β-lactam amide linkage by aβ-lactamase, a yellow colored solution containing Cefesone or Nitrocefin turns red as a result of the formation of a highly conjugated compound. Thus, determination of.β-lactamase activity, of utmost importance in clinical microbiology, is easily achieved in a visual manner. The application of Cefesone has been described by Sutton et al.[1] Although a synthetic approach was given by the authors, no experimental details were disclosed. To our knowledge, the synthesis of Nitrocefin has only been performed by workers from the Glaxo Laboratories.[2] Following this approach, Nitrocefin can be obtained starting from the diphenylmethyl ester of the 3′-phosphonium bromide of Cephalothin in 45% yield.

In this paper we present a straightforward synthesis of Cefesone and Nitrocefin starting from readily available *tert*-butyl (1*S*,6*R*,7*R*)-3-bromomethyl-1-oxo-7-(phenylacetamido)ceph-3-em-4-carboxylate[3] (3-BMC™).

When a suspension of 3-BMC™ in MeOAc/MeOH is treated with 1.5 equivalents of triphenylphosphine, the corresponding 3′-phosphonium bromide **2** is obtained as white crystals in a yield of 93%. The 7-(2-bromo-2-phenylacetamido) derivative of 3-BMC™, present in small quantities in commercial samples, is also converted into the desired product. The latter phenomenon does not occur in the absence of MeOH. The debromination reaction is in accordance with the observation of alcohol-promoted dehalogenation of α-carbonyl compounds with triphenylphosphine.[4]

In the next step, **2** is reduced with 1.5 equivalents of phosphorus trichloride and subsequently coupled with 2,4-dinitrobenzaldehyde. After purification, the 3′-alkenylcephalosporin **3** is obtained in 83% yield and consists of a 1:12 mixture of *E*- and *Z*-isomers.

Deprotection of the *tert*-butyl ester is achieved using relatively mild acidic conditions. Lewis acid promoted hydrolysis of cephalosporin esters is a procedure already reported for benzyl,[5] diphenylmethyl,[6] and 4-methoxybenzyl[7] esters using aluminum trichloride.[8]The use of tin tetrachloride[8] and titanium tetrachloride[5, 8] has also been advocated. However, a high yielding Lewis acid mediated method for deprotection of cephalosporin *tert*-butyl esters has not been reported thus far. In our laboratories we developed a procedure to hydrolyze *tert*-butyl esters of cephalosporins with titanium tetrachloride.[9] Thus, upon treatment of **3** with titanium tetrachloride, the β-lactamase indicator Cefesone (**4**) can be obtained after workup and crystallization in 63% yield as the single *E*-isomer with a purity of 99%. It should be noted that during titanium tetrachloride mediated ester hydrolysis, almost complete *Z* to *E* isomerization takes place.

The phenylacetyl side chain in 4 can be replaced by a 2-thienylacetyl side chain to produce Nitrocefin (**6**). Various methods exist for removal of the phenylacetyl function. Chemical deacylation[3,10] using silyl carboxyl protection, PCl_5 mediated imide chloride formation, and subsequent alcoholysis, is a commonly accepted method in many synthetic schemes giving satisfactory yields in most examples. However, this procedure employs toxic chemicals and solvents at sub-zero temperatures. Fortunately, an attractive alternative may be found in enzymatic hydrolysis using penicillin acylase (EC 3.5.1.11) under mild conditions. Thus, the phenylacetyl side chain is removed using immobilized penicillin-G acylase in water at neutral pH.[11] The nucleus **5** is obtained by precipitation at pH 3 in 77% yield.

In the final step, the 2-thienylacetyl side chain is coupled in a two-step process. Compound **5** is silylated using 1,3-bis(trimethylsilyl)urea and in the next step 2-thiopheneacetyl chloride is added. After workup and crystallization from CH_2Cl_2, Nitrocefin is obtained as the single *E*-isomer in 67% yield with a purity of 95%.

The above-mentioned procedure enabled us to reproducibly prepare the chromogenic cephalosporin Cefesone in 49% yield from 3-BMC™ on a multigram scale. In two additional steps, Nitrocefin can be prepared in 52% yield.

All reagents were of commercial quality. $TiCl_4$ was purchased from Aldrich. 1,3-Bis(trimethylsilyl)urea, 2-thiopheneacetyl chloride and PPh_3 were purchased from Acros. PCl_3 was purchased from Merck. 3-BMC™ was from Gist-brocades (industrial quality). 2,4-Dinitrobenzaldehyde, required for the introduction of the 2,4-dinitrophenyl moiety, was synthesized in 48% yield from 2,4-dinitrotoluene (Acros) as follows: according to the procedure developed by Kalir for the synthesis of 2-nitrobenzaldehyde,[12] *N*-(2,4-dinitrobenzyl)pyridinium bromide was obtained from 2,4-dinitrotoluene after bromination with NBS in the presence of benzoyl peroxide followed by reaction with pyridine. Reaction with *N*,*N*-dimethyl-4-nitrosoaniline yielded *N*-(4-dimethylaminophenyl)-α-2,4-dinitrophenyl nitrone as reported by Behr and coworkers.[13] The nitrone was converted into the desired aldehyde using the Kröhnke method.[14] Immobilized penicillin-G acylase[15]

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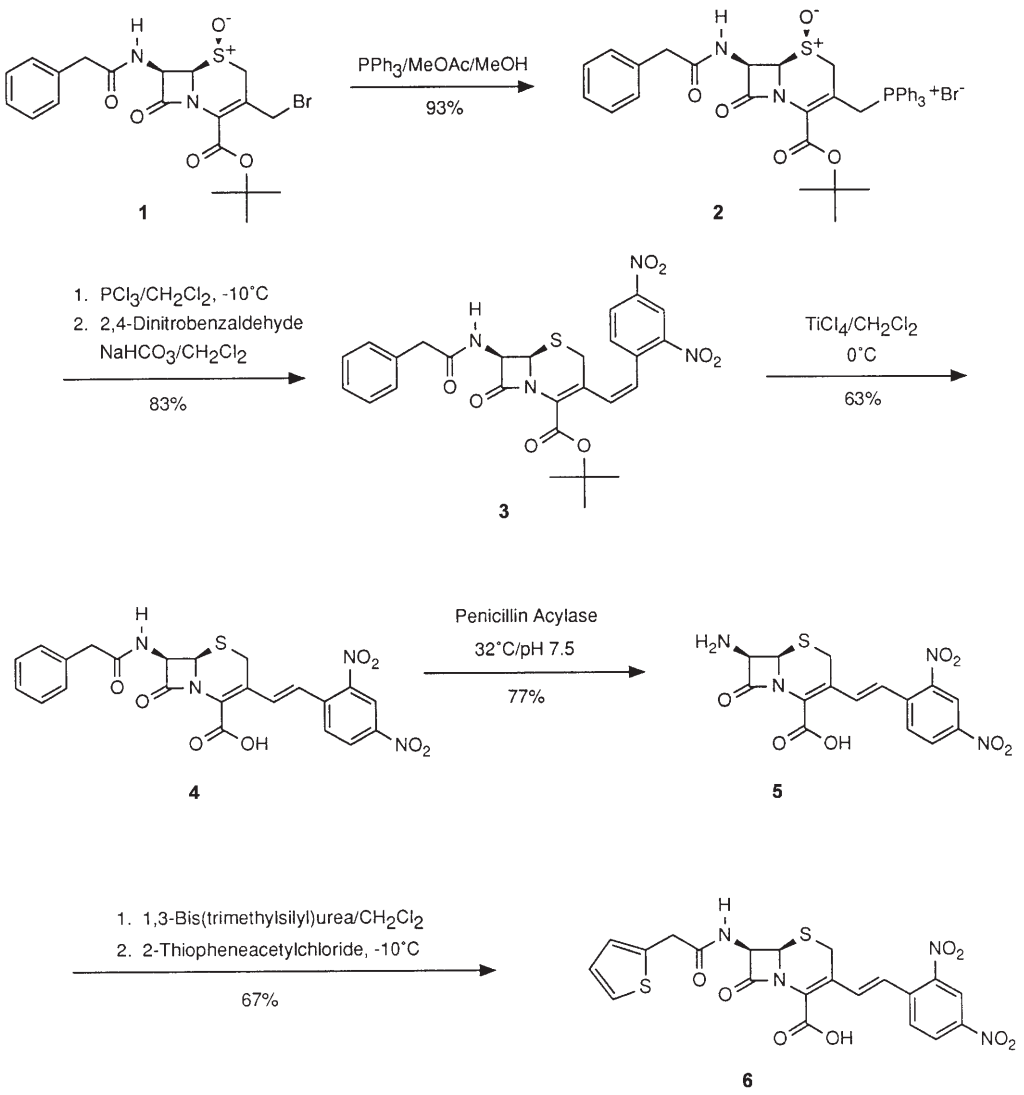

(150 units/gram; 1 unit corresponds to the amount of enzyme that hydrolyzes per minute 1 μmol of penicillin-G potassium salt at a concentration of 100 g L^{-1} in 0.05 M potassium phosphate buffer at pH 8.0 and 28 °C) was repeatedly washed with water before use and weighed without drying. Mass spectra were obtained with an AMD 402 mass spectrometer. ^{1}H spectra were recorded on a Bruker AM 360 MHz instrument. Purities were determined with ^{1}H spectroscopy using an internal reference.

***tert*-Butyl (1*S*,6*R*,7*R*)-1-Oxo-7-(phenylacetamido)-3-(triphenylphosphoniomethyl)ceph-3-em-4-carboxylate Bromide (2):**

A suspension of **1** {52.14 g, purity 87.3%, 94.2 mmol, contaminated with 6.3% of the 7-[2-bromo-2-(phenylacetamido)] derivative, 5.8 mmol} and PPh_3 (39.74 g, purity 99%, 150 mmol) in MeOAc (50 mL) and MeOH (10 mL) was boiled under reflux for 1 h. MeOAc (875 mL) was added in 1,5 h and reflux was continued for 2 h. The mixture was cooled to 0 °C and stirred for 1.5 h. The white crystals were collected by filtration, washed with MeOAc (250 mL) and dried at 40 °C under reduced pressure for 12 h to give 74.12 g (purity 93.9%, 93.3 mmol; 93%) of **2.**

IR (KBr): ν = 3380 (NH), 1790 (CO, β-lactam), 1705 (CO, ester), 1680 (CO, amide), 1030 cm^{-1} (SO).

FABMS: *m/z* = 665.2 (M^+-Br).

^{1}H NMR ($CDCl_3$; 360 MHz): δ = 1.23 (s, 9H, *tert*-butyl), 3.20 (d, 1H, J = 19.1 Hz, H_2), 3.57 (s, 2H, $ArCH_2$), 4.87 (dd, 1H, J_1 = 5.5 Hz, J_2 = 19.1 Hz, H_2), 5.03 (d, 1H, J = 4.3 Hz, H_6), 5.16 (dd, 1H, J_1 = 14.0 Hz, J_2 = 17.7 Hz, CH_2P), 5.41 (dd, 1H, $J_1 = J_2$ = 14.0 Hz, CH_2P), 6.02 (dd, 1H, $J_{6,7}$ = 4.3 Hz, $J_{7,NH}$ = 10.1 Hz, H_7), 6.75 (d, 1H, J = 10.1 Hz, NH), 7.2–7.7 (m, 20H, ArH).

***tert*-Butyl (6*R*,7*R*)-3-(2,4-Dinitrostyryl)-7-(phenylacetamido)-ceph-3-em-4-carboxylate, *Z*-Isomer (3):**

A stirred solution of **2** (39.72 g, purity 93.9%, 50.0 mmol) in CH_2Cl_2 (125 mL) was cooled under N_2 to –20°C. PCl_3 (6.55 mL, 75.1 mmol) was added at such a rate that the temperature never exceeded –10°C. After stirring for 1 h at –10°C, the solution was cooled to –55 °C and slowly added to ice water (250 mL) under vigorous stirring while maintaining the pH of the aqueous phase at 4.0 with 4 M NaOH. The organic phase was separated and extracted with ice water (250 mL). The combined aqueous phases were back-extracted with CH_2Cl_2 (75 mL). The combined organic phases were concentrated at 20°C under reduced pressure to a volume of 250 mL. To this solution was added a solution of 2,4-dinitrobenzaldehyde (29.41 g, purity 95%, 142.5 mmol) in CH_2Cl_2 (1 L) and 1 M aq $NaHCO_3$ (1240 mL). After vigorous stirring for 2 h, the phases were separated and the organic phase was extracted with brine (500 mL), dried ($MgSO_4$), and concentrated under reduced pressure to a volume of 100 mL. The solution of crude **3** thus obtained was purified by short-column chromatography on silica gel (565 g) using CH_2Cl_2 with a 0 to 3% gradient of acetone as elution solvent. The appropriate fractions, as judged by TLC (CH_2Cl_2/acetone 10:1, v/v), were pooled and concentrated under reduced pressure to give 25.43 g (purity 7% *E*-isomer, 85% *Z*-isomer, 41.3 mmol; 83%) of **3** as an orange foam.

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IR (KBr): ν = 3290 (NH), 1785 (CO, β-lactam), 1715 (CO, ester), 1665 (CO, amide), 1525 cm^{-1} (NO_2).

MS (DCI): *m/z* = 584.2 (MNH_4^+).

^1H NMR ($CDCl_3$; 360 MHz): δ = 1.53 (s, 9H, *tert*-butyl), 2.75 (d, 1H, *J* = 18.2 Hz, H_2), 3.25 (d, 1H, *J* = 18.2 Hz, H_2), 3.63 (ABq, 2H, *J* = 15.9 Hz, $ArCH_2$), 4.91 (d, 1H, *J* = 4.9 Hz, H_6), 5.84 (dd, 1H, $J_{6,7}$ = 4.9 Hz, $J_{7,NH}$ = 9.1 Hz, H_7), 6.02 (d, 1H, *J* = 9.1 Hz, NH), 6.84 (ABq, 2H, *J* = 11.9 Hz, CH=CH), 7.3 (m, 5H, ArH), 7.57 (d, 1H, *J* = 8.6 Hz, ArH), 8.88 (d, 1H, *J* = 2.3 Hz, ArH).

(6*R*,7*R*)-3-(2,4-Dinitrostyryl)-7-(phenylacetamido)ceph-3-em-4-carboxylic Acid, *E*-Isomer (4):

A stirred solution of **3** (67.41 g, purity 8% *E*-isomer, 81% *Z*-isomer, 105.9 mmol) in CH_2Cl_2 (1685 mL) was cooled to –25 °C. $TiCl_4$ (53 mL, 482 mmol) was added in 10 min and the temperature was brought to 0°C. After 1.75 h, chilled 2 M HCl (1685 mL) was added at such a rate that the temperature remained under 10 °C. The organic phase was separated and extracted with 2 M HCl (2 × 1685 mL), H_2O (1685 mL) and brine (1685 mL). The organic phase was concentrated under reduced pressure to give an orange foam. Crude **4** thus obtained was crystallized by dissolving in acetone (1350 mL) at 65 °C and adding H_2O (675 mL). Crystallization was allowed to proceed for 16 h at 0°C and the crystals were collected by filtration. Recrystallization of the product was performed by dissolving the material in acetone/HOAc (2:1) at 53 °C, removing the solvent (1700 mL) by evaporation under reduced pressure, and stirring for 16 h at 20 °C. Crystals were collected by filtration, washed with HOAc (300 mL) and Et_2O (250 mL), and dried under vacuum at 45 °C to give 34.33 g (purity 99% *E*-isomer, 66.6 mmol; 63%) of **4** as yellow crystals.

IR (KBr): ν = 3300 (NH), 1780 (CO, β-lactam), 1715 (CO, amide), 1625 (CO, carboxylic acid), 1525 cm^{-1} (NO_2).

MS (DCI): *m/z* = 528.0 (MNH_4^+).

^1H NMR ($CDCl_3$/DMSO-d_6, 1:2; 360 MHz): δ = 3.50/3.58 (ABq, 2H, *J* = 14.1 Hz, $ArCH_2$), 3.62/3.77 (ABq, 2H, *J* = 17.5 Hz, H_2), 5.05 (d, 1H, *J* = 4.9 Hz, H_6), 5.72 (dd, 1H, $J_{6,7}$ = 4.9 Hz, $J_{7,NH}$ = 8.3 Hz, H_7), 7.3 (m, 6H, ArH + CH=C), 7.63 (d, 1H, *J* = 16.1 Hz, C=CH), 7.82 (d, 1H, *J* = 8.8 Hz, ArH), 8.33 (dd, 1H, J_1 = 2.1 Hz, J_2 = 8.8 Hz, ArH), 8.66 (d, 1H, *J* = 2.1 Hz, ArH), 8.97 (d, 1H, *J* = 8.3 Hz, NH).

(6*R*,7*R*)-3-(2,4-Dinitrostyryl)-7-aminoceph-3-em-4-carboxylic Acid, *E*-Isomer (5):

Compound **4** (521 mg, purity 98% *E*-isomer, 1.00 mmol) was dissolved in water (20 mL) at pH 7.5 by the addition of 0.1 M NaOH. The temperature of the solution was brought to 32 °C and immobilized penicillin-G acylase (1 g) was added. The pH was kept at 7.5 by the continuous addition of 0.1 M NaOH using an automatic titration apparatus. After 3 h, when NaOH consumption had stopped, the immobilized enzyme was removed by filtration and the pH of the red solution was brought to 3.0. After stirring for 1 h, orange crystals were collected by filtration, washed with H_2O (25 mL), and dried under vacuum at 40 °C. Yield: 359 mg (purity 75% *E*-isomer, 9% *Z*-isomer, 0.77 mmol; 77%).

IR (KBr): ν = 1800 (CO, β-lactam), 1610 (CO, carboxylic acid), 1535 cm^{-1} (NO_2).

MS (DCI): *m/z* = 410. 1 (MNH_4^+).

^1H NMR (DMSO-d_6; 360 MHz): δ = 3.67/3.74 (ABq, 2H, *J* = 17.5 Hz, H_2), 4.87 (d, 1H, *J* = 5.1 Hz, H_6/H_7), 5.10 (d, 1H, *J* = 5.1 Hz, H_7/H_6), 7.18 (d, 1H, *J* = 16.1 Hz, CH=C), 7.51 (d, 1H, *J* = 16.1 Hz, C=CH), 7.95 (d, 1H, *J* = 8.7 Hz, ArH), 8.47 (dd, 1H, J_1 = 2.1 Hz, J_2 = 8.7 Hz, ArH), 8.70 (d, 1H, *J* = 2.1 Hz, ArH).

(6*R*,7*R*)-3-(2,4-Dinitrostyryl)-7-(2-thienylacetamido)ceph-3-em-4-carboxylic Acid, *E*-Isomer (6):

A suspension of **5** (392 mg, purity 82% *E*-isomer, 0.82 mmol) in CH_2Cl_2 (100 mL) was evaporated under reduced pressure to a volume of 25 mL. 1,3-Bis(trimethylsilyl)urea (216 mg, 1.06 mmol) and ammonium bromide (1 mg) were added and the mixture was boiled under reflux for 1.5 h. The red solution was cooled to –12°C and 2-thiopheneacetyl chloride (0.14 mL, purity 99%, 1.13 mmol) was added. After stirring for 1.5 h at –10 °C, CH_2Cl_2 (1.5 L) and H_2O (125 mL) were added and the pH of the aqueous phase was adjusted to 3.0 with 0.1 M NaOH. The organic phase was extracted with H_2O (3 × 500 mL), dried (Na_2SO_4), and concentrated under reduced pressure to a volume of 25 mL. The yellow crystals formed were collected by filtration, washed with CH_2Cl_2 (25 mL), and dried in a desiccator over P_2O_5. Yield: 299 mg (purity 95% *E*-isomer, 0.55 mmol; 67%).

IR (KBr): ν = 3295 (NH), 1780 (CO, β-lactam), 1720 (CO, amide), 1630 (CO, carboxylic acid), 1525 cm^{-1} (NO_2).

MS (DCI): *m/z* = 534.1 (MNH_4^+).

^1H NMR ($CDCl_3$/DMSO-d_6; 360 MHz): δ = 3.57/3.70 (ABq, 2H, *J* = l7.4 Hz, H_2), 3.78 (ABq, 2H, *J* = 15.9 Hz, thiophene-CH_2), 5.00 (d, 1H, *J* = 4.9 Hz, H_6), 5.77 (dd, 1H, $J_{6,7}$ = 4.9 Hz, $J_{7,NH}$ = 8.4 Hz, H_7), 6.89 (m, 2H, thiophene-H), 7.15 (m, 2H, CH=C + thiophene-H), 7.67 (d, 1H, *J* = 16.1 Hz, C=CH), 7.76 (d, 1H, *J* = 8.8 Hz, ArH), 8.27 (dd, 1H, J_1 = 2.0 Hz, J_2 = 8.8 Hz, ArH), 8.45 (d, 1H, *J* = 8.4 Hz, NH), 8.67 (d, 1H, *J* = 2.0 Hz, ArH).

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