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An Improved Synthesis of CENTA, a Chromogenic Substrate for β-Lactamases

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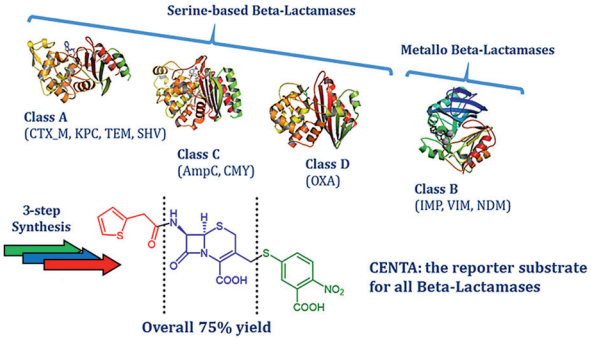

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Abstract 7-β-Thien-2-yl-acetamido-3-[(4-nitro-3-carboxyphenyl)thiomethyl]-3-cephem-4-carboxylic acid (CENTA) is a yellow chromogenic β-lactamases (BL) substrate. It hydrolyses readily in the presence of all BL and is therefore suitable for kinetic studies, the detection of BL enzymes in crude extracts and chromatographic fractions. CENTA is commercially available at a high price because of the cumbersome synthetic protocol, the only currently available for its preparation. Here we describe a new efficient and improved process for the preparation of CENTA. Starting from the easily available 7-aminocephalosporanic acid (7-ACA) through a three-step synthesis, CENTA was obtained with a 75% overall yield. The newly developed process proceeds through a pivotal intermediate in cephalosporin chemistry, which may be used as starting compound for the development of new cephalosporin derivatives.

Key words chromogenic substrate, β-lactamase kinetics, β-lactamase detection, synthesis, CENTA

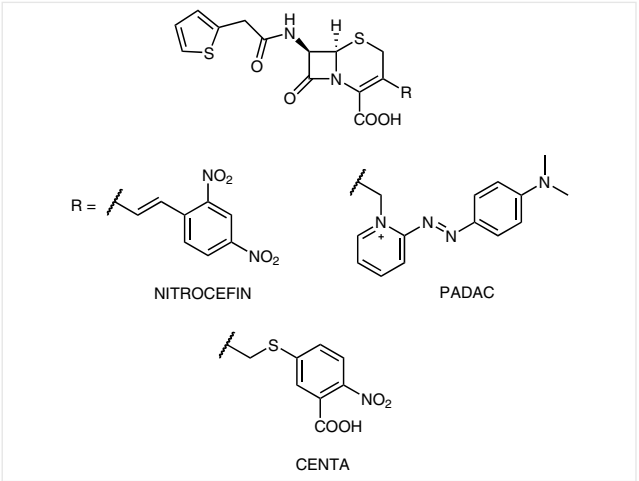
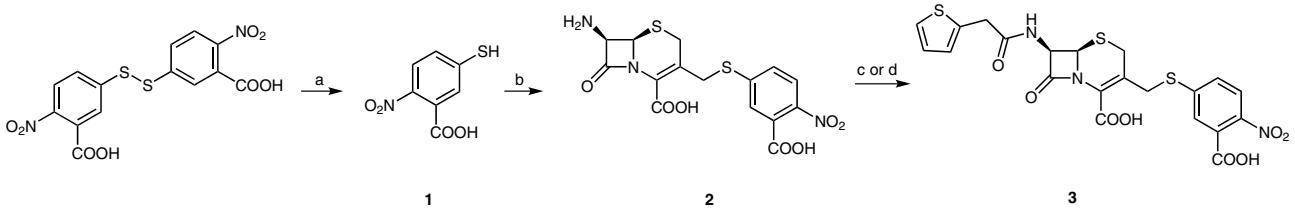


Figure 1 Chemical structures of chromogenic substrates for β-lactamases: nitrocefin, PADAC and CENTA

Over the years several chromogenic substrates for the rapid qualitative and quantitative detection of β-lactamases (BL), their kinetic characterization and for the development of BL inhibitors have been developed.[1–10] These substrates, such as (2*S*,5*R*,6*R*)-6-{[carboxy(phenyl)acetyl]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (nitrocefin) and pyridine-2-azo-4′-(*N*′,*N*′-dimethylaniline)cephalosporin (PADAC, Figure 1), change their color when hydrolyzed by BL allowing direct observation on agar plates or in the test solution. However, nitrocefin is an expensive commercially available substrate,[7b] while PADAC is no longer on sale.

In this context, the 7-β-thien-2-yl-acetamido-3-[(4-nitro-3-carboxyphenyl)thiomethyl]-3-cephem-4-carboxylic acid (CENTA) represents a valid alternative as β-lactamase-labile, chromogenic cephalosporin reporter substrate (Figure 1). It undergoes color change from light yellow (λ_{max} ca. 340 nm) to chrome yellow (λ_{max} ca. 405 nm) in concomitance with the hydrolysis of its β-lactam ring. CENTA has been used extensively for BL kinetic studies, for their early detection,[11–18] and for the screening of new potential β-lactamase inhibitors.[2,19–21]

In comparison with other available chromogenic derivatives, CENTA has shown an effective and comparable diagnostic profile for the detection of several classes of β-lactamases including class B (metallo-β-lactamases) and it is more efficient than nitrocefin vs. class D β-lactamases.[2,12] In addition, CENTA is easily handled: it is more stable under air, to direct light exposure, in TRIS buffer solution, and in serum; its stock solution can be stored for a longer time compared to nitrocefin.[7,22–24]



1 2 3

Scheme 1 *Reagents and conditions*: a) $NaBH_4$ (8 equiv), 80% EtOH (v/v), 0 °C to r.t., 97%; b) 7-ACA (1 equiv), BF_3 (3 equiv), MeCN, r.t., 2 h, 93%; c) 2-thienylacetyl chloride (1 equiv), potassium trimethylsilanolate (2 equiv), MeCN, r.t. 1 h, then 50 °C, 2 h, 83%; d) 2-(thiophen-2-yl)acetic acid (1 equiv), EDC·HCl (1.1 equiv), anhyd DMF, N_2, 0 °C 0.5 h, then r.t., 6 h, 73%.

At present, only one synthesis of CENTA is reported in the literature, developed by Bebrone et al.[11a] Following this first report, only two other research groups reported the in-house synthesis of CENTA slightly modifying the original synthetic procedure.[2,19] The above-mentioned synthetic route for CENTA includes the cleavage of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by dithiothreitol to yield 5-mercapto-2-nitrobenzoic acid (TNB) and the subsequent reaction with commercially available cephalothin in water for six hours. This procedure, however, suffers low yield, employs expensive commercial reagents, and requires a laborious protocol.[11b]

Here we describe a high-yielding, three-step synthesis and for the first time a full physical-chemical characterization of CENTA starting from readily available 7-aminocephalosporanic acid (7-ACA) and DTNB and avoiding the need of classical carboxylic group protection in position 3 of the cephem ring (Scheme 1).

Our synthetic procedure allowed the preparation of intermediate (6*R*,7*R*)-7-amino-3-{[(3-carboxy-4-nitrophenyl)thio]methyl}-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid) (**2**) which was easily converted into CENTA (**3**). Although the herein described new protocol required one more synthetic step than the original procedure, it allows the preparation of CENTA in higher yield and purity and passes through intermediate **2** that could represent, by derivatization of the nitrogen in position 7, a promising pivotal intermediate in the design and development of future chromogenic cephalosporins.

As depicted in Scheme 1, TNB (**1**) was prepared by $NaBH_4$-mediated reduction of commercial DTNB in a freshly prepared solution of $NaBH_4$ in ethanol in 97% yield.[25] This was a significant improvement on the previously developed synthetic procedure where the reduction of the disulfide bridge represented the yield-limiting step.[2,12,19] Thiol **1** was then reacted with 7-ACA in anhydrous acetonitrile (MeCN) in the presence of boron trifluoride to afford derivative **2** in 93% yield.[26] With respect to the synthesis of CENTA described in the literature,[11a] the use of a polar aprotic solvent such as MeCN resulted in higher stability of the cephem nucleus. Moreover, in accordance with Saikawa et al.,[27] employment of a Lewis acid improved the solubility of 7-ACA and allowed the fine-tuning of the nucleophilic displacement by decreasing the nucleophilicity of the amino group in 7-ACA and, at the same time, by rendering the acetoxy group a better leaving group through coordination. Several Lewis acids were tested: while $SnCl_4$, $ZnCl_2$, F_3CSO_3H, and H_2SO_4 gave poor results, BF_3 allowed the achievement of high yield.

Two different synthetic approaches were explored to convert the intermediate **2** into CENTA. In the first method, **2** was directly reacted with 2-(thiophen-2-yl)acetyl chloride in anhydrous MeCN using potassium trimethylsilanolate as a base to yield CENTA (**3**) in 83% yield. Trimethylsilanolate is an organic-solvent-soluble base, which reduces the amount of aqueous solution employed during the purification step.[28a] In the second attempt, EDC·HCl was used as condensing agent between **2** and the commercially available 2-(thiophen-2-yl)acetic acid in anhydrous DMF, and **3** was obtained in 73% yield.[28b]

In conclusion, an efficient synthesis of CENTA starting from the readily available 7-ACA and following a straightforward and inexpensive synthetic route was developed. The synthetic route described herein does not require classical protection at the carboxylic group of the cephem ring, fastening the process, and avoids water as solvent for the key nucleophilic displacement. In fact, considering the instability of the cephem nucleus in aqueous solution,[27] we envisaged first of all that the overall yield would improve by performing the substitution reaction under nonaqueous conditions, as here demonstrated. In addition, the synthetic approach allows easy access to cephalosporin intermediate **2**, which may be used as starting compound for the development of new cephalosporin derivatives. CENTA was prepared in three steps in overall 75% yield (method c) or 66% yield (method d) with respect to the Bebrone et al.[11a] synthetic procedure that allows to obtain CENTA in overall 44% yield. Considering that the total cost for the herein reported procedure is less than 100€ per gram, any biological laboratory, with access to chemistry facilities, could easily prepare CENTA in-house.

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Supporting Information

Supporting information for this article is available online at http://dx.doi.org/10.1055/s-0035-1562454.

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(25) **5-Mercapto-2-nitrobenzoic Acid (TNB, 1)**
In a 50 mL one-neck round-bottom flask, 5,5′-dithiobis(2-nitrobenzoic acid) (420 mg, 1.06 mmol, 1 equiv) was slurried in 80% EtOH (v/v, 5 mL) cooled to 0 °C with an ice bath. $NaBH_4$ (320 mg, 8.46 mmol, 8 equiv) was dissolved in distilled water (2 mL) and slowly added dropwise through an addition funnel (CAUTION! Vigorous effervescence develops!). The resulting dark red mixture was stirred at r.t. until gas evolution subsided. After dilution with EtOH (5 mL) and distilled water (5 mL), the mixture was acidified with 2 N HCl, extracted with CH_2Cl_2 (3 × 20 mL), and the pooled organic layers were washed with distilled water (3 × 50 mL) and brine (3 × 50 mL). The yellow organic solution was dried over Na_2SO_4, filtered, and concentrated to dryness to afford the desired thiol as a bright orange solid (410 mg, 97% yield); mp 143–145 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.84 (1 H, s, SH), 5.77 (1 H, br, COOH), 7.48 (1 H, dd, *J* = 8.5, 2.1 Hz, H-4), 7.63 (1 H, d, *J* = 2.1 Hz, H-6), 7.86 (1 H, d, *J* = 8.5 Hz, H-3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 125.2 (C-3), 126.6 (C-1), 128.8 (C-6), 128.9 (C-4), 141.6 (C-5), 146.5 (C-2), 165.3 (COOH). MS: *m/z* = 200.1 $[M + H]^+$; 198.0 $[M - H]^-$. Anal. Calcd for $C_7H_5NO_4S$: C, 42.21; H, 2.53; N, 7.03. Found: C, 42.19; H, 2.55; N, 7.07.
(26) **(6*R*,7*R*)-7-Amino-3-{[(3-carboxy-4-nitrophenyl)thio]-methyl}-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (2)**
7-Aminocephalosporanic acid (7-ACA, 0.547 g, 2.01 mmol, 1 equiv) and 5-mercapto-2-nitrobenzoic acid (**1**, 0.400 g, 2.01 mmol, 1 equiv) were successively added to a stirred solution of BF_3 (1 M solution in THF, 6.03 mL, 6.03 mmol, 3 equiv) in 40 mL anhydrous MeCN, and the resulting solution was stirred at r.t. for 2 h. After cooling in an ice-bath, the mixture was diluted with water (50 mL) and adjusted to pH 4.0 by addition of 28% NH_4OH. The resulting ochre-yellow precipitate was collected by filtration and washed with water and acetone to afford the title compound as a light ochre powder (0.768 g, 93%); mp 184 °C (dec.); $[\alpha]_D^{25}$ = –26.4 (1.07% w/v in DMSO). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.49 (1 H, d, *J* = 17.8 Hz, H-4), 3.69 (1 H, d, *J* = 17.8 Hz, H-4), 4.16 (1 H, d, *J* = 12.8 Hz, CH_2–C-3), 4.29 (1 H, d, *J* = 12.8 Hz, CH_2–C-3), 4.77 (1 H, d, *J* = 5.0 Hz, H-7), 4.98 (1 H, d, *J* = 5.0 Hz, H-6), 7.62 (1 H, dd, *J* = 8.6, 1.9 Hz, H-6′), 7.66 (1 H, d, *J* = 1.9 Hz, H-2′), 7.93 (1 H, d, *J* = 8.6 Hz, H-5′). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 27.2 (C-4), 35.2 (*C*H$_2$–C-3), 59.4 (C-6), 63.5 (C-7), 124.5 (C-3)*, 125.0 (C-5′), 126.8 (C-2)*, 128.0 (C-2′), 130.2 (C-6′), 144.7 (C-1′), 145.1 (C-4′), 163.8 (C-2–*C*OOH), 166.4 (C-3′–*C*OOH), 169.4 (C-8) [* assignments may be interchangeable]. MS: *m/z* = 412.1 $[M + H]^+$; 410.0 $[M - H]^-$. Anal. Calcd for $C_{15}H_{13}N_3O_7S_2$: C, 43.79; H, 3.18; N, 10.21. Found: C, 43.76; H, 3.17; N, 10.24.
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(28) **(6*R*,7*R*)-3-{[(3-Carboxy-4-nitrophenyl)thio]methyl}-8-oxo-7-]2-(thiophen-2-yl)acetamido]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (CENTA, 3)**
(a) **Method c**
A solution of potassium trimethylsilanolate (0.218 g, 1.7 mmol, 2 equiv) and 2-thienylacetyl chloride (0.136 g, 0.850 mmol, 1 equiv) in anhydrous MeCN (10 mL) was added simultaneously to a suspension of **2** (0.35 g, 0.850 mmol, 1 equiv) in anhydrous CH_2Cl_2 (30 mL) at 0 °C over 0.5 h. The resulting mixture was

stirred at r.t. for 1 h and then heated at 50 °C for 2 h. After evaporation of the solvent under reduced pressure, the residue was taken up with 10 mL of distilled water, acidified to pH 2.0 with 1 M HCl, and extracted with EtOAc (3 × 15 mL). The pooled organic layers were washed with water (3 × 50 mL) and brine (3 × 50 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The residue was recrystallized from MeOH–Et_2O to give the desired compound in undissociated form as a yellow solid (0.377 g, 0.705 mmol, 83% yield with respect to **2**)

(b) **Method d**

In a 50 mL round-bottom flask, compound **2** (0.35 g, 0.850 mmol, 1 equiv) and 2-(thiophen-2-yl)acetic acid (0.120 g, 0.850 mmol, 1 equiv) were dissolved in anhydrous DMF (5 mL) at 0 °C under nitrogen atmosphere. A solution of EDC·HCl (0.180 g, 0.935 mmol, 1.1 equiv) in DMF (5 mL) was added dropwise at 0 °C, and the mixture was allowed to warm gradually and left to stir at r.t. for 6 h. The solvent was then concentrated in vacuo, and water (20 mL) was added. The resulting aqueous phase was extracted with EtOAc (3 × 20 mL) and the pooled organic layers washed with water (3 × 50 mL), brine (3 × 50 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Subsequently, the dry residue was dissolved in 25 mL of water containing 1 equiv of $NaHCO_3$. The solution was filtered through a Sephadex G-10 column and freeze-dried to afford the sodium salt of the title compound as a light brown solid (0.327 g, 73% yield with respect to **2**); mp 59.3–62.2 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.54 (1 H, d, *J* = 18.0 Hz, H-4), 3.73 (1 H, d, *J* = 18.0 Hz, H-4), 3.76 (2 H, AB system, CH_2–C-2′), 4.26 (2 H, s, CH_2–C-3), 5.12 (1 H, d, *J* = 4.8 Hz, H-6), 5.66 (1 H, dd, *J* = 4.8, 8.3 Hz, H-7), 6.93 (1 H, m, H-3′), 6.95 (1 H, d, *J* = 5.0 Hz, H-4′), 7.36 (1 H, dd, *J* = 1.2, 5.0 Hz, H-5′), 7.66 (1 H, dd, *J* = 2.1, 8.6 Hz, H-6″), 7.70 (1 H, d, *J* = 2.1 Hz, H-2″), 7.96 (1 H, d, *J* = 8.6 Hz, H-5″), 9.12 (1 H, d, *J* = 8.3 Hz, NH–C-7), 13.7–13.8 (2 H, br, 2 × COO*H*). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 27.6 (C-4), 35.1 (*C*H_2–C-3), 36.2 (*C*H_2–C-2′), 58.2 (C-6), 59.6 (C-7), 125.0 (C-5″), 125.4 (C-5′), 126.1 (C-2)*, 126.3 (C-3)*, 126.8 (C-3′), 127.1 (C-4′), 128.0 (C-2″), 129.5 (C-3″), 130.2 (C-6″), 137.3 (C-2′), 144.9 (C-1″), 145.1 (C-4″), 163.2 (C-2–*C*OOH), 165.0 (C-8), 166.2 (C-3″–*C*OOH), 170.4 (CONH) [* assignments may be interchangeable]. MS: *m/z* = 536.2 $[M + H]^+$; 534.0 $[M - H]^-$. Anal. Calcd for $C_{21}H_{17}N_3O_8S_3$: C, 47.10; H, 3.20; N, 7.85. Found: C, 47.12; H, 3.18; N, 7.85.