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Synthesis of pyochelin–norfloxacin conjugates

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Abstract—Using synthetic functionalized analogues of pyochelin, a siderophore common to several pathogenic *Pseudomonas* and *Burkholderia* species, four fluoroquinolone-pyochelin conjugates were efficiently synthesized and evaluated for their biological activities.

Pseudomonas aeruginosa and *Burkholderia cepacia*, two opportunistic Gram-negative bacteria, are causes of severe and often lethal lung infections especially for cystic fibrosis affected or immunocompromised patients.[1] The low outer membrane permeability of these bacteria and the antibiotic mediated selective pressure raised an increasing number of multiresistant strains. Therefore, it appears necessary to develop new therapeutic strategies against these microorganisms.

In this context, iron uptake pathways developed by *P. aeruginosa* and *B. cepacia* might become interesting targets for new antibiotics against these two pathogenic bacteria. Indeed, during infection, the bacterial proliferation requires high quantities of various nutrients. Among them iron is one of the most crucial. Although this metal is abundant in the earth crust, its bioavailability is limited by the low solubility of iron(III) at physiological pHs. Moreover, even if higher eukaryotes contain substantial amounts of this crucial element, the latter is so tightly associated with transport and storage proteins that it is not freely available for pathogens. Consequently, the level of free iron in biological fluids is usually estimated to only 10^{-18} M. To overcome this vital problem, pathogenic microorganisms synthesize and excrete molecules called siderophores, able to chelate iron(III) and compete with the host for this element.[2] In Gram-negative bacteria, the siderophore-iron(III) complex is recognized by a specific outer membrane receptor and then transported into the bacterial cell by a process driven by the proton motive force of the inner membrane.[3,4] The iron uptake pathways can be used in Trojan horse strategy in order to counter the low outer membrane permeability of *P. aeruginosa* and *B. cepacia*.[5,6]

A few years ago we used pyoverdine, the chromopeptidic siderophore of *P. aeruginosa*, as a vector to transport a fluoroquinolone into the bacterial cells. The corresponding adducts were shown to be transported inside the cells and showed promising bactericidal activity.[6] However this strategy was of limited application since each *P. aeruginosa* strain produces its own pyoverdine along with a specific transporter. Moreover, very few cross-feeding have been observed between these different pyoverdines and their corresponding transporters. In this respect and since all *P. aeruginosa* strains and almost all *B. cepacia* genomovars excrete a common siderophore, the pyochelin **1** (Fig. 1),[7,8] this latter siderophore

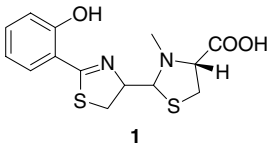

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Figure 1. Structure of pyochelin **1**.

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appeared to be more suitable to vectorize antibiotics the more so as synthetic pyochelin as well as its functionalized analogues are now readily available.[9]

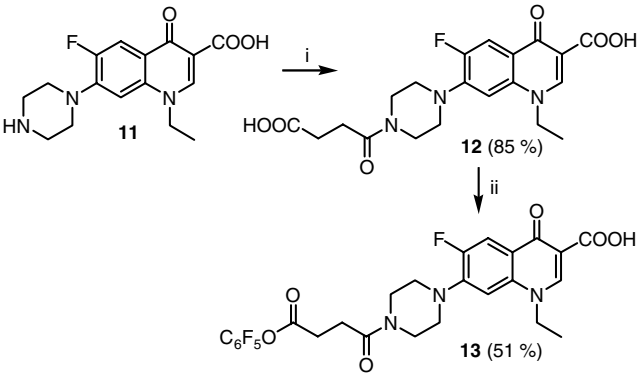
The present article describes the synthesis of pyochelin–norfloxacin conjugates and the first evaluation of their bactericidal activities.

The phenol and the carboxylate function of pyochelin **1** are known to be required for iron chelation and recognition by FptA receptor.[10,11] Therefore, these two chemical functions appeared to be not suitable for the conjugation with an antibiotic. In order to minimize interactions with these crucial groups, we synthesized and described three pyochelin analogues, functionalized with a Teoc protected amine function in position 5 of the aromatic ring (Scheme 1: compounds **2**, **3**, and **4**).[9a] This new function was further used to connect the synthetic pyochelin to an antibiotic fitted with a spacer arm. The next step was thus the deprotection of the three pyochelin analogues **2**, **3**, and **4**. Unfortunately, the Teoc group removal using the most common fluoride ion sources was unsuccessful,[12,13] leading generally to complex mixtures predominantly composed of starting material. To overcome this situation, three new functionalized pyochelins **5**,[9b] **6**, and **7**, whose amine function is protected with a *tert*-butyloxycarbonyl (Boc) group, were synthesized. The protecting group removal on molecules **5**, **6**, and **7** appeared to be much easier: in the presence of 6% TFA in dichloromethane, the corresponding ammonium salts **8**, **9**, and **10** were obtained very efficiently. A higher proportion of TFA or a prolonged reaction time led often to complex mixtures mainly composed of degradation products (Scheme 1).

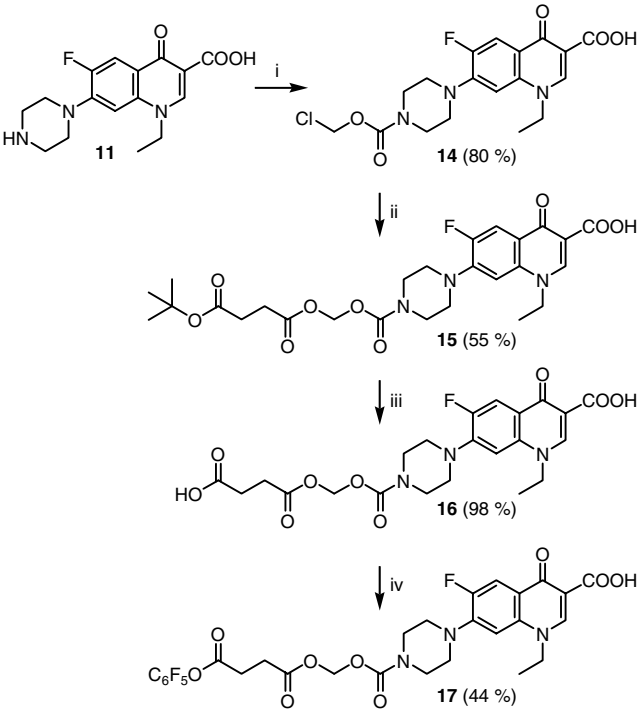
The compounds **8**, **9**, and **10** were not very stable and were therefore used as quickly as possible in the coupling reaction with the pentafluorophenyl esters of the antibiotic-linker units. The antibiotic chosen in a first approach was norfloxacin **11**, a fluoroquinolone active against *P. aeruginosa* that acts by inhibiting the bacterial topoisomerase.[14] The spacer arms used to link the drug to the functionalized pyochelins were of two types: a succinic linker, stable in physiological condition, and a labile one which could release the norfloxacin under physiological conditions.

The synthesis of the fluoroquinolone fitted with a stable spacer arm starts with the condensation of norfloxacin **11** with succinic anhydride.[6] The free succinic carboxylate of the resulting diacid **12** was then regioselectively converted to its pentafluorophenyl ester **13** using EDCI (Scheme 2).[15,16]

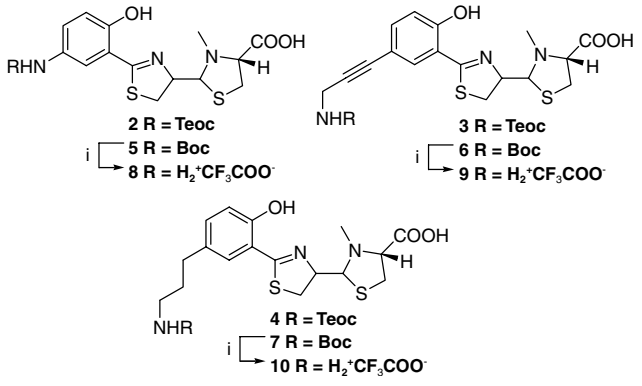
In the case of the synthesis of the labile spacer arm-antibiotic block, norfloxacin **11** was first reacted with chloromethylchloroformate and gave compound **14** with



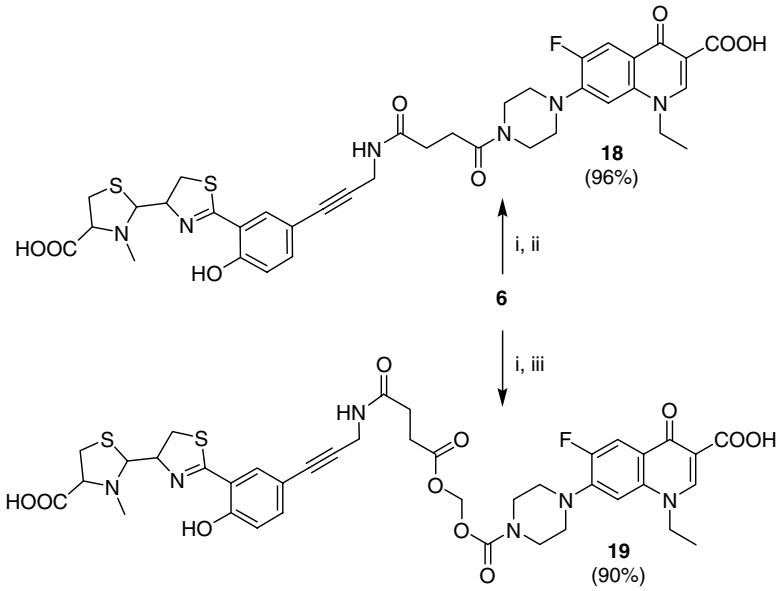
Scheme 2. Synthesis of the norfloxacin–succinic spacer arm block **13**. Reagents and conditions: (i) succinic anhydride, pyridine, DMSO, 95 °C; (ii) pentafluorophenol, EDCI, dioxane, 60 °C.



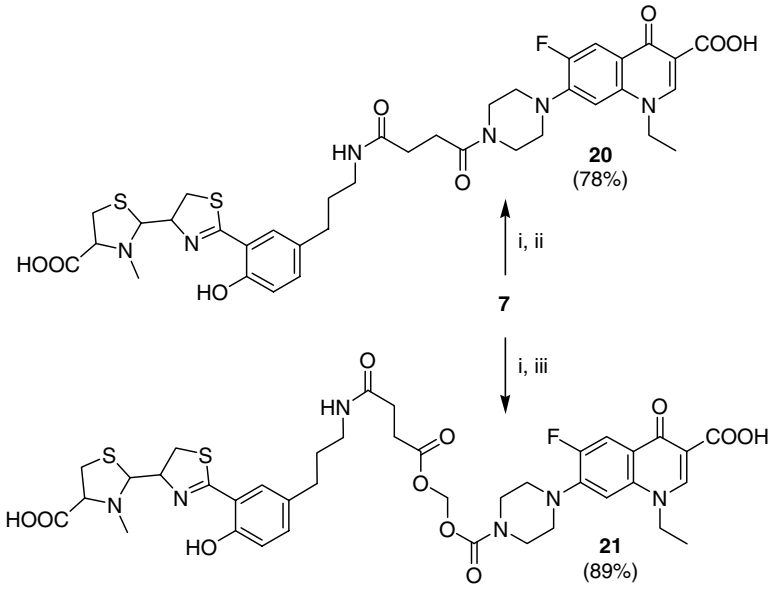
Scheme 3. Synthesis of the norfloxacin-hydrolyzable spacer arm block **17**. Reagents and conditions: (i) chloromethylchloroformate, 1,8-bis(*N*,*N*-dimethylamino)naphthalene, $CHCl_3$, 20 °C; (ii) succinic acid mono-*tert*-butyl ester, Ag_2CO_3, DMF, 95 °C; (iii) TFA, CH_2Cl_2, 20 °C; (iv) EDCI, pentafluorophenol, dioxane, 20 °C.



Scheme 1. The nine functionalized synthetic pyochelins **2**, **3**, **4** (Teoc protected), **5**, **6**, **7** (Boc-protected), and **8**, **9**, **10** (ammonium salts). Reagents and conditions: (i) TFA, CH_2Cl_2, 20 °C.



Scheme 4. Synthesis of prodrugs **18** and **19**. Reagents and conditions: (i) TFA, CH_2Cl_2, 20 °C; (ii) **13**, DIPEA, dry DMF, 20 °C; (iii) **17**, DIPEA, dry DMF, 20 °C.



Scheme 5. Synthesis of prodrugs **20** and **21**. Reagents and conditions: (i) TFA, CH_2Cl_2, 20 °C; (ii) **13**, DIPEA, dry DMF, 20 °C; (iii) **17**, DIPEA, dry DMF, 20 °C.

an excellent yield. The reaction of chloride **14** with *tert*-butyl hemisuccinate in the presence of silver carbonate gave the corresponding *tert*-butyl ester derivative **15**.[17] This acylation method produced the clean desired compound **15** easily and efficiently, and was preferred to the previously published method.[6] The *tert*-butyl protecting group was removed with TFA and the resulting free carboxylic acid **16** was then converted to the activated ester **17** (Scheme 3).[18]

The coupling reactions between functionalized pyochelins **9** and **10**, and activated esters **13** and **17** were carried out in dry DMF in the presence of DIPEA.[19] The conjugates **18** and **19** containing the acetylenic extension were obtained from **6** in 96% and 90% yield, respectively, over two steps (Scheme 4).

Similarly, the conjugates **20** and **21** containing the aliphatic extension were isolated from **7** in 78% and 89% yield, respectively, over two steps (Scheme 5).

Unfortunately we were not able to isolate the conjugates derived from the pyochelin analogue **5**. The amine function of the intermediate **8** seemed to be not nucleophilic enough to react efficiently with activated esters **13** or **17**.

As a preliminary biological evaluation, the four pyochelin–norfloxacin adducts were then tested against

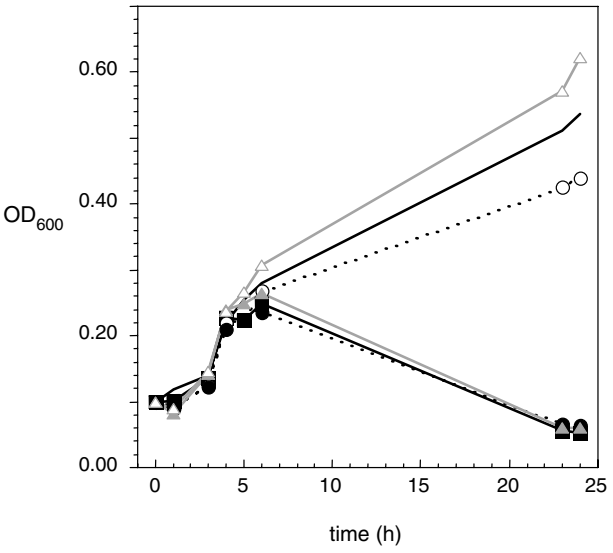


Figure 2. Growth of wild-type *P. aeruginosa* in the presence of the prodrugs.[20] *P. aeruginosa* PAO1 cells at an OD_{600} of 0.1 were incubated at 30 °C in succinic medium (black line),[21] or in the presence of 10 μM norfloxacin (■, black line) conjugate **18** (○, dotted line), conjugate **19** (●, dotted line), conjugate **20** (△, grey line) or conjugate **21** (▲, grey line) for 25 h. The cell growth was followed at 600 nm. All growth experiments were performed at least in triplicate.

P. aeruginosa PAO1 strain (Fig. 2). Only the labile-arm conjugates **19** and **21** showed a lethal activity against *P. aeruginosa* as pronounced as for norfloxacin. For the stable arm-containing conjugates **18** and **20**, the growth curves showed no significant inhibition. Apparently the dissociation of the siderophore-antibiotic conjugate is necessary for observing a bactericidal activity of these conjugates. Therefore the norfloxacin, when bound to pyochelin by a non-hydrolyzable spacer arm, may be either not able to reach the DNA gyrase in the cytoplasm or is too sterically hindered by the siderophore moiety to interact and inhibit this crucial enzyme. These results are quite similar to those obtained previously with conjugates derived from the siderophore pyoverdine.[6]

In summary, we have synthesized four unprecedented pyochelin–norfloxacin conjugates. The preliminary biological tests against a wild-type strain of *Pseudomonas aeruginosa* show a bactericidal activity for two of these four conjugates. Further studies are under way in our laboratory in order to investigate if these compounds are Trojan horse conjugates transported by the pyochelin uptake pathway or if these conjugates behave as classical antibiotic prodrugs. In addition, complementary biological evaluations with different pathogenic clinical CF strains of *Pseudomonas aeruginosa* and *Burkholderia cepacia* will be investigated.

Acknowledgments

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16. *Synthesis of activated ester* **13***:* Diacid **12**[6] (96 mg, 0.23 mmol) and pentafluorophenol (84 mg, 0.27 mmol) were dissolved in dioxane (2 mL). The solution was cooled at 0 °C and EDCI (52 mg, 0.27 mmol) was added. The reaction mixture was heated to 60 °C for 4 h and then the solution was allowed to cooled down to room temperature and evaporated to dryness. The crude product was purified by chromatography on a silica gel column (CH_2Cl_2 to CH_2Cl_2/MeOH:95/5), to give product **13** (68 mg, 0.12 mmol, 51%) as a white powder. R_f 0.29 (Acetone/AcOEt/AcOH:50/48/2); ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) = 8.68 (s, 1H); 8.10 (d, J = 12.8 Hz, 1H); 6.71 (d, J = 7.0 Hz, 1H); 4.32 (q, J = 7.2 Hz, 2H); 3.91–3.88 (m, 2H); 3.77–3.74 (m, 2H); 3.55–3.38 (m, 2H); 3.30–3.27 (m, 2H); 3.09 (t, J = 6.5 Hz, 2H); 2.83 (t, J = 6.5 Hz, 2H); 1.59 (t, J = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) = 177.1; 169.3; 169.0; 167.2; 153.6 (d, J = 250 Hz); 147.4, 145.7 (d, J = 10 Hz); 137.1,

121.2 (d, J = 7 Hz); 113.1 (d, J = 23 Hz); 108.5; 104.4; 50.3; 50.2; 49.9; 49.6; 49.5; 28.7; 27.9; 14.6; ESI-TOF m/z 586 ($M+H^+$).

17. *Synthesis of compound* **15***:* Halide **14** (300 mg, 0.73 mmol) was dissolved in DMF (7.8 mL) and then succinic acid mono-*tert*-butyl ester (254 mg, 1.46 mmol) and silver carbonate (603 mg, 2.19 mmol) were added successively. The reaction mixture was heated to 95 °C for 4 h and then the solution was cooled down to room temperature and was filtered through Celite. Celite was thoroughly washed with a mixture CH_2Cl_2/MeOH:90/10 and the resulting filtrate evaporated to dryness. After adsorption on silica, the crude residue was purified by chromatography on a silica gel column (CH_2Cl_2 to CH_2Cl_2/MeOH:96/4). After crystallization from CH_2Cl_2/cyclohexane, compound **15** (220 mg, 0.40 mmol, 55%) was obtained as a white powder. R_f 0.74 (CH_2Cl_2/MeOH:95/5); ^1H NMR (DMSO-d_6, 300 MHz): δ (ppm) = 8.96 (s, 1H); 7.94 (d, J = 13.2 Hz, 1H); 7.21 (d, J = 7.1 Hz, 1H); 5.73 (s, 2H); 4.59 (q, J = 7.1 Hz, 2H); 3.61 (br s, 4H); 3.35 (br s, 4H); 2.58–2.43 (m, 4H); 1.41 (t, J = 7.1 Hz, 3H); 1.37 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) = 176.8; 171.5; 171.2; 167.0; 153.4 (d, J = 252 Hz); 153.4; 147.2; 145.7 (d, J = 10 Hz); 137.1; 120.7 (d, J = 7 Hz); 112.6 (d, J = 23 Hz); 108.2; 104.4; 80.9; 80.5; 49.8; 49.6; 43.9; 43.5; 30.0; 29.2; 28.1; 14.5.
18. *Synthesis of activated ester* **17***:* To a solution of *tert*-butylic ester **15** (99 mg, 0.18 mmol) in dichloromethane (7 mL), TFA (3 mL) was added. After 3 h at 23 °C, the mixture was evaporated to dryness and the residue was triturated in diethyl ether. After filtration, compound **16** (87 mg, 0.18 mmol, 98%) was obtained as a white powder and was used without any further purification. Diacid **16** (50 mg, 0.10 mmol) and pentafluorophenol (37 mg, 0.20 mmol) were dissolved in dioxane (1 mL). The solution was cooled to 0 °C and EDCI (23 mg, 0.12 mmol) was added. The reaction mixture was stirred for 4 h at 24 °C and then the solution was evaporated to dryness. The crude product was purified by chromatography on a silica gel column (CH_2Cl_2 to CH_2Cl_2/MeOH:95/5) and gave product **17** (29 mg, 0.05 mmol, 44%) as a white powder. R_f 0.51 (CH_2Cl_2/MeOH:95/5); ^1H NMR ($CDCl_3$, 300 MHz): δ (ppm) = 8.69 (s, 1H); 8.10 (d, J = 12.8 Hz, 1H); 6.83 (d, J = 6.8 Hz, 1H); 5.85 (s, 2H); 4.31 (q, J = 7.3 Hz, 2H); 3.73 (br s, 4H); 3.28 (br s, 4H); 3.03 (dd, J = 5.3; 6.0 Hz, 2H); 2.85 (dd, J = 5.3; 6.0 Hz, 2H); 1.59 (t, J = 7.3 Hz, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) = 177.1; 170.7; 168.4; 167.3; 159.9; 153.6 (d, J = 250 Hz); 153.4; 147.4; 145.8 (d, J = 10 Hz); 137.1; 121.2 (d, J = 7 Hz); 113.1 (d, J = 23 Hz); 108.5; 104.4; 80.8; 49.9; 49.7; 43.9; 43.6; 28.8; 28.2; 14.6; ESI-TOF m/z 660 ($M+H^+$) 682 ($M+Na^+$) 698 ($M+K^+$).
19. *General procedure for norfloxacin–pyochelin conjugates* **18***,* **19***,* **20***,* **21***:* To a solution of Boc-protected amine **6** (or **7**) (0.10 mmol) in dichloromethane (5 mL), TFA (0.30 mL) was added successively. After 3 h at room temperature, the mixture was evaporated to dryness and the residue was triturated in diethyl ether. After filtration, compound **9** (or **10**) was obtained as yellow powder which was used without any further purification. Compound **9** (or **10**) was taken up in dry DMF (5 mL) and activated ester **13** (or **17**) (0.10 mmol) and DIPEA (0.15 mmol) was added. After 18 h at room temperature (20–24 °C), the reaction mixture was evaporated to dryness and then triturated in diethyl ether. After filtration, the desired conjugate **18** (96%), **19** (90%), **20** (78%) or **21** (89%) was obtained. Compound (**18**) ESI-TOF m/z 779 ($M+H^+$); HRMS Calcd for $C_{37}H_{40}FN_6O_8S_2$: 779.2328. Found 779.2336. Compound (**19**) ESI-TOF m/z 853 ($M+H^+$); HRMS Calcd for $C_{39}H_{42}FN_6O_{11}S_2$: 853.2332. Found 853.2331. Compound (**20**) ESI-TOF m/z 783 ($M+H^+$); HRMS Calcd for $C_{37}H_{44}FN_6O_8S_2$: 783.2641. Found 783.2640. Compound (**21**) ESI-TOF m/z 857 ($M+H^+$); HRMS Calcd for $C_{39}H_{46}FN_6O_{11}S_2$: 857.2645. Found 857.2648.
20. Bacterial strains and culture conditions: *P. aeruginosa* PAO1 cells were grown overnight in a succinate medium at 30 °C.[21] For measurements of bacterial growth, an overnight culture was diluted to an OD_{600} of 0.1 in succinate medium and was incubated at 30 °C in the presence of either 10 μM norfloxacin, or of prodrugs **18**, **19**, **20** or **21** for 25 h. The cell growth was followed at 600 nm.
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