



## Synthesis of a (+)-anatoxin-a analogue for monoclonal antibodies production

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### ABSTRACT

Anatoxin-a analogue was prepared by resolution of aminoacid 1 using chiral (*R*)-4-phenyl-oxazolidin-2-thione as derivatizing agent. X-ray diffraction of a diastereomer allowed us to determine its absolute configuration. The synthesis could then be completed in few steps followed by introduction of an amide linker bearing a terminal alkyne in order to attach a carrier protein for monoclonal antibodies production.

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Anatoxin-a is a highly toxic secondary amine alkaloid produced by cyanobacteria (blue green algae). Anatoxin's toxicity relies on its ability to bind to nicotinic acetylcholine receptors (agonist) with a lethal dose LD<sub>50</sub> of 0.2–0.5 mg/kg IP in mice, it is also designed as Very Fast Death Factor (VFDF). The presence of this toxin in drinking waters is responsible for water poisoning, thus creating a major threat on public health and making detection and quantification of anatoxin-a in water a big issue.<sup>1</sup> For such a purpose, monoclonal antibody technology can be very useful for the development of a detection assay. The production of monoclonal antibodies that are able to bind to anatoxin requires the immunization of mice with an hapten composed of a protein (usually BSA or KLH) and the toxin or a close analogue. In order to focus mAbs recognition onto this small hapten structure, we decided to use the extracyclic ketone as the anchor for the attachment of the carrier protein. Yet, two key structural features on anatoxin-a are detrimental towards the commonly used techniques for the bio-conjugation of this small hapten onto carrier proteins: the key secondary and reactive amine (thus any attachment using activated esters or Schiff base formation are inappropriate), and the presence of an activated alpha-beta unsaturated ketone, preventing the use of soft nucleophiles such as thiols. We thus decided to undertake the synthesis of an anatoxin analogue **12** that will be attached onto the carrier protein through click chemistry (Fig. 1).<sup>2</sup>

Among those bioorthogonal bio-conjugation means, the [3+2] Huisgen cycloaddition reaction is by far the most used and docu-

mented reaction,<sup>3</sup> thereafter, to attach the analogue with the protein by click chemistry, we decided to functionalize the analogue with a terminal alkyne and link this alkyne to the anatoxin core by an amide function. In order to have the partner azide function on the carrier protein, BSA will be modified prior to the bio-conjugation with readily obtained activated ester of 6-azido hexanoic acid (which could be obtained in one step from commercially available 6-bromohexanoic acid).

In order to stress the immunological response on the bicyclic core and hence the mab recognition, we chose to replace the methylketone of anatoxin by a disubstituted amide, thus preventing the planar arrangement of the C=O and the intracyclic double bond in order to (1) have substantial difference in the side chain compared to anatoxin and (2) lower the toxicity of the hapten caused by an irreversible linkage to nicotinic acetylcholine receptors through 1,4-addition on the  $\alpha,\beta$ -unsaturated carbonyl function.

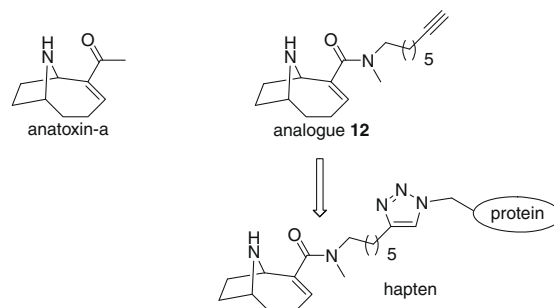


Figure 1. Anatoxin-a and analogue.

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Anatoxin-a possesses an azabicyclo-[4.2.1] nonane ring system that has led to many racemic and enantioselective approaches.<sup>4</sup> Among these approaches, the construction of the azabicyclo-[4.2.1] nonane ring starting from a cyclooctene ring by aminocyclisation is one of the most popular but leads usually to racemic syntheses.<sup>4c,i,5</sup> Recently Fülöp described a deracemisation of a cyclooctene fused  $\beta$ -lactam by enzymatic resolution but did not complete the synthesis.<sup>6</sup> We herein report on the construction of the azabicyclo-[4.2.1] nonane ring by resolution of racemic aminoacid using chiral auxiliaries such as oxazolidin-2-thiones and the completion of the synthesis of an anatoxin analogue.

The racemic  $\beta$ -aminoacid **1** was readily prepared from the corresponding  $\beta$ -lactam after methanolysis, Boc-protection of the amine and saponification. Then resolution was done by derivatization of the acid with chiral (*R*)-4-phenyl-oxazolidin-2-thione and separation of diastereomers **3a** and **3b** by simple chromatography on silica (Scheme 1). Fortunately, we were able to obtain X-ray structure of diastereomer **3b** that allowed us to determine the absolute configurations of the aminoacid moiety (Fig. 2).<sup>7</sup>

Having identified isomer **3b** as 1*R*,8*S* isomer, we selected isomer **3a** (1*S*,8*R*) to continue the synthesis of anatoxin analogue, as the amino group has the right (*R*) absolute configuration. Thus, **3a** was converted into its methyl ester **4** (imidazole, MeOH and DMAP cat.) and subjected to selenium-assisted aminocyclisation, giving the azabicyclo-[4.2.1] nonane **5** as a single regioisomer<sup>4i</sup> in 75% yield. Instead of removing the selenium residue by oxidative elimination followed by hydrogenolysis (low yielding), we found that Raney Nickel smoothly converted **5** into its reduced derivative **6** in a quantitative yield, thus greatly improving the overall yield of the transformation (Scheme 2).

Starting from ester **6**, two different strategies were assessed in order to access the targeted unsaturated amide **12**.<sup>8</sup> The first one envisioned the early formation of the amide before the introduction of the unsaturation, whereas the second one relied on the early introduction of the unsaturation before forming the amide bond (Scheme 3).

Following the first way, after saponification of ester **6** to carboxylic acid **7**, amide **8** was obtained by coupling with secondary amine **13** in the presence of coupling agent PyBrOP<sup>8</sup> in 54% yield (no reaction occurred with DCC). Secondary amine **13** was prepared in 5 steps from octynol after silylation of the alkyne, mesylation and iodination of the hydroxyle followed by displacement with methylamine (29% overall yield). Unfortunately, all attempts to introduce the unsaturation by  $\alpha$ -phenylselenenylation of amide **8** proved to be unsuccessful. Indeed, enolization at  $-78^\circ\text{C}$  followed by addition of PhSeBr led only to the recovery of starting material, whereas reaction performed at  $0^\circ\text{C}$  gave only degradation products. Therefore, we turned our attention to the second strategy.

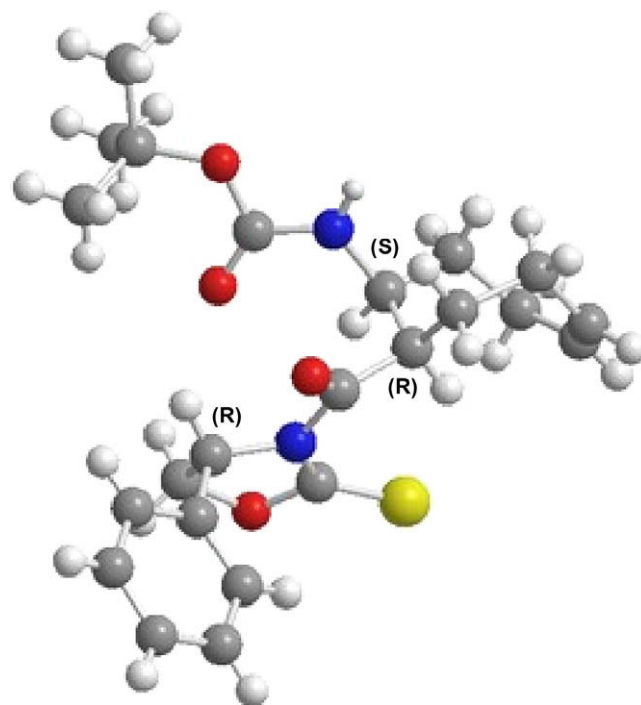
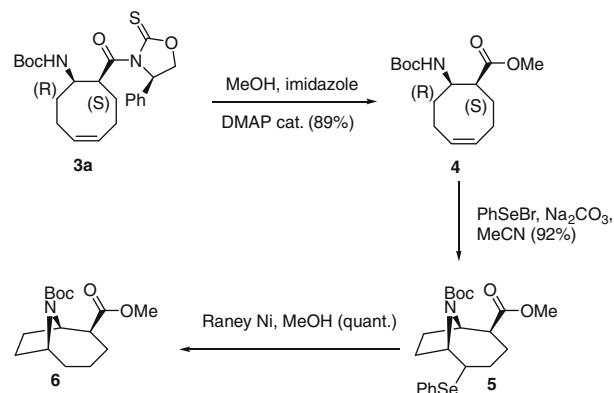
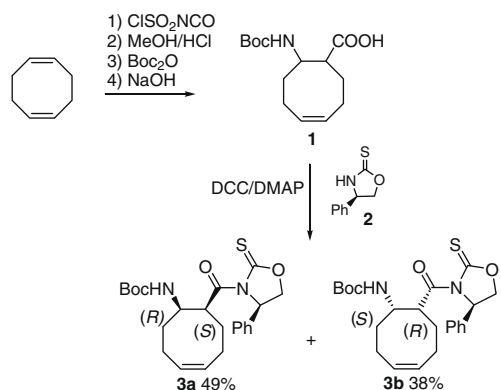


Figure 2. X-ray structure of diastereomer **3b**.



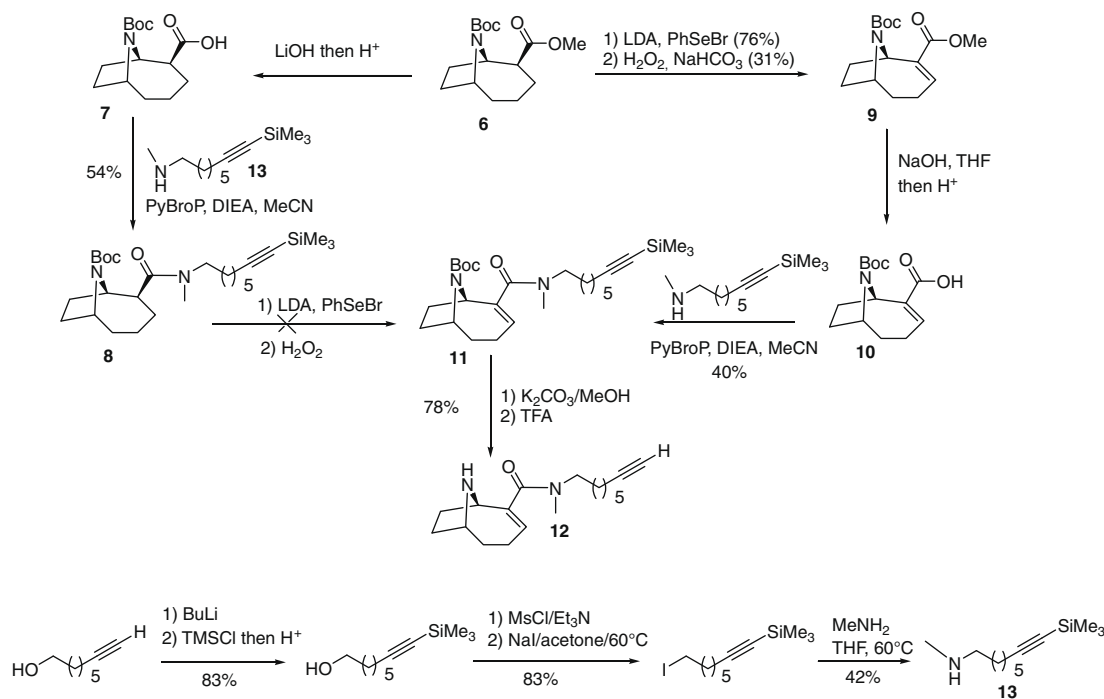
Scheme 2. Synthesis of ester **6**.



Scheme 1. Resolution of the racemic aminoacid **1**.

Introduction of the unsaturation was thus performed prior to the introduction of the amide side chain.  $\alpha$ -phenylselenenylation of **6** (LDA and PhSeBr) followed by oxidative elimination ( $\text{H}_2\text{O}_2$  and  $\text{NaHCO}_3$ ) gave ester **9** in a modest 25% yield over these two steps (low yields were always obtained for  $\beta$ -elimination whatever the conditions). Saponification of the methyl ester yielded the carboxylic acid **10** in almost quantitative yield. Introduction of the amide function proved again to be difficult and only PyBrOP<sup>8</sup> allowed us to obtain **11** in a modest 40% yield. Thereafter, targeted anatoxin-a analogue **12** could be obtained after desilylation and Boc deprotection through TFA treatment in 78% yield for the last two steps (Scheme 3).<sup>9–11</sup>

The synthesis of (+)-anatoxin-a analogue **12** has been accomplished by resolution of racemic aminoacid **1** and selenium-assisted cyclisation. Its terminal alkyne function will allow the linkage with a protein before production of monoclonal antibodies. This last step is now under way and the results will be reported in due course.

Scheme 3. Synthesis of (+)-anatoxin-a analogue **12**.

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- <sup>1</sup>H NMR data of compound **12** are similar to literature data concerning anatoxin,<sup>10</sup> except mainly for H-1 which suffers from a shielding due to the presence of the amide moiety as its carbonyl is not planar with the internal C=C bond. The broadness of the signals is also due to the presence of this moiety.
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- Spectral data of selected compounds*: Diastereomers **3a** and **3b** were separated by column chromatography on silica using cyclohexane/EtOAc (8:2) as the solvent.  
Compound **3a**: (*R*<sub>f</sub> = 0.27); <sup>1</sup>H NMR: (300 MHz, CDCl<sub>3</sub>) δ 7.41–7.28 (m, 5H), 5.76–5.58 (m, 3H), 5.14 (br d, *J* = 7.3 Hz, 1H), 4.77 (t, *J* = 9.0 Hz, 1H), 4.43 (m, 1H), 4.38 (dd, *J* = 9.0, 4.5 Hz, 1H), 4.27 (m, 1H), 2.50–2.40 (m, 2H), 2.20–2.03 (m, 3H), 1.94–1.78 (m, 3H), 1.35 (s, 9H); <sup>13</sup>C NMR: (75 MHz, CDCl<sub>3</sub>) δ 185.2,

175.5, 154.6, 138.1, 130.1, 129.2, 128.9, 128.7, 126.0, 79.2, 73.7, 62.4, 50.0, 44.2, 33.1, 28.4, 26.5, 25.8 23.7; IR (CH<sub>2</sub>Cl<sub>2</sub>): ν 3428, 2932, 2360, 1714, 1694, 1504, 1455, 1367, 1161, 1086, 1019, 737, 699 cm<sup>-1</sup>; MS (ESI): *m/z* 431 ([M+H]<sup>+</sup>), 453 ([M+Na]<sup>+</sup>); [α]<sub>D</sub><sup>20</sup> = -84.8 (c 1.0, CHCl<sub>3</sub>).

Compound **3b**: (*R*<sub>f</sub> = 0.36); <sup>1</sup>H NMR: (300 MHz, CDCl<sub>3</sub>) δ 7.30–7.16 (m, 5H), 5.70–5.62 (m, 1H), 5.57–5.49 (m, 1H), 5.41 (br d, *J* = 7.3 Hz, 1H), 5.09 (br d, *J* = 11.3 Hz, 1H), 4.79 (t, *J* = 8.4 Hz, 1H), 4.70 (d, *J* = 9.9 Hz, 1H), 4.31 (dd, *J* = 8.4, 2.2 Hz, 2H), 3.50–2.20 (m, 2H), 2.12–1.81 (m, 3H), 1.72–1.52 (m, 3H), 1.37 (s, 9H); <sup>13</sup>C NMR: (75 MHz, CDCl<sub>3</sub>) δ 186.0, 175.7, 155.4, 139.4, 131.5, 129.2, 128.6, 127.2, 126.0, 79.5, 74.3, 63.8, 51.5, 43.4, 33.4, 30.2, 28.5, 26.0, 25.0; IR (KBr): ν 3386, 2975, 2940, 2888, 1691, 1654, 1515, 1367, 1152, 974 cm<sup>-1</sup>; MS (ESI): *m/z* 431 ([M+H]<sup>+</sup>); [α]<sub>D</sub><sup>20</sup> = 21.2 (c 1.0, CHCl<sub>3</sub>).

Compound **4**: <sup>1</sup>H NMR: (300 MHz, CDCl<sub>3</sub>) δ 5.73–5.57 (m, 2H), 5.03 (br d, *J* = 8.2 Hz, 1H), 4.14–4.09 (m, 1H), 3.70 (s, 3H), 2.84–2.82 (m, 1H), 2.49–2.42 (m, 1H), 2.30–2.21 (m, 1H), 2.18–2.08 (m, 2H), 2.07–1.93 (m, 1H), 1.89–1.71 (m, 3H), 1.41 (s, 9H); <sup>13</sup>C NMR: (75 MHz, CDCl<sub>3</sub>) δ 174.8, 155.1, 130.5, 129.3, 79.3, 51.7, 50.2, 48.1, 33.0, 28.5, 27.4, 24.5, 23.5; IR (CH<sub>2</sub>Cl<sub>2</sub>): ν 3372, 2934, 2360, 1714, 1694, 1504, 1455, 1366, 1249, 1170 cm<sup>-1</sup>; MS (ESI) *m/z* 284 ([M+H]<sup>+</sup>); [α]<sub>D</sub><sup>20</sup> = +55.7 (c 1.0, CHCl<sub>3</sub>).

Compound **6** (2 rotamers 66:34): <sup>1</sup>H NMR: (300 MHz, CDCl<sub>3</sub>) δ 4.69 (d, *J* = 8.8 Hz, 0.34H), 4.52 (d, *J* = 9.2 Hz, 0.66H), 4.35 (d, *J* = 8.8 Hz, 0.66H), 4.21–4.13 (m, 0.34H), 3.70 (s, 1H), 3.68 (s, 2H), 2.47–2.29 (m, 2H), 2.10–1.64 (m, 6H), 1.62–1.53 (m, 3H), 1.42 (s, 3H), 1.39 (s, 6H); <sup>13</sup>C NMR: (75 MHz, CDCl<sub>3</sub>) δ 175.0 (mino), 174.8 (majo), 153.5, 79.6 (majo), 79.1 (mino), 57.2 (mino), 56.8 (majo), 56.3, 53.3, 52.1 (mino), 51.7 (majo), 35.5 (majo), 34.1 (mino), 33.7 (mino), 32.8 (majo), 28.6 (mino), 28.5 (majo), 27.0, 26.8 (mino), 26.5 (majo), 21.9 (majo), 21.8 (mino); IR (CH<sub>2</sub>Cl<sub>2</sub>): ν 3367, 2934, 1738, 1693, 1404, 1173, 1112 cm<sup>-1</sup>; MS (ESI): *m/z* 284 ([M+H]<sup>+</sup>); [α]<sub>D</sub><sup>20</sup> = -30.7 (c 1.07, CHCl<sub>3</sub>).

Compound **9** (2 rotamers 70:30): <sup>1</sup>H NMR: (300 MHz, CDCl<sub>3</sub>) δ 6.97 (t, *J* = 5.9 Hz, 1H), 5.14 (d, *J* = 7.5 Hz, 0.3H), 5.02 (d, *J* = 8.8 Hz, 0.7H), 4.40–4.38 (m, 0.7H), 4.31–4.22 (m, 0.3H), 3.73 (s, 3H), 2.42–2.23 (m, 2H), 2.19–2.00 (m, 3H), 1.78–1.53 (m, 3H), 1.44 (s, 2.7H), 1.39 (s, 6.3H); <sup>13</sup>C NMR: (75 MHz, CDCl<sub>3</sub>) δ 167.3 (majo), 167.1 (mino), 153.3 (majo), 153.2 (mino), 141.9 (majo), 141.5 (mino), 140.7 (majo), 138.4 (mino), 79.4, 55.7 (majo), 55.4 (mino), 55.1 (mino), 54.7 (majo), 52.0, 32.7 (mino), 31.7 (majo), 31.1 (mino), 30.5 (majo), 30.0, 28.8 (mino), 28.6 (majo), 24.0; IR (CH<sub>2</sub>Cl<sub>2</sub>): ν 3372, 2975, 1714, 1694, 1404, 1366, 1237, 1170, 1111, 770 cm<sup>-1</sup>; MS (ESI) *m/z* 282 ([M+H]<sup>+</sup>); [α]<sub>D</sub><sup>20</sup> = -18.4 (c 1.0, CHCl<sub>3</sub>).

Compound **10** (2 rotamers 55:45): <sup>1</sup>H NMR: (300 MHz, CDCl<sub>3</sub>) δ 7.12 (t, *J* = 6.0 Hz, 0.55H), 6.88 (t, *J* = 6.1 Hz, 0.45H), 5.03 (d, *J* = 8.5 Hz, 0.55H), 4.91 (d, *J* = 8.3 Hz, 0.45H), 4.42 (br d, *J* = 8.5 Hz, 0.55H), 4.38–4.30 (m, 0.45H), 2.46–2.41 (m, 2H), 2.35–2.04 (m, 2H), 2.00–1.58 (m, 4H), 1.46 (s, 5H), 1.41 (s, 4H); <sup>13</sup>C NMR: (75 MHz, CDCl<sub>3</sub>) δ 171.4, 154.3 (mino), 153.3 (majo), 144.5, 141.9 (majo), 140.1 (mino), 80.7 (majo), 79.7 (mino), 56.4 (mino), 55.7 (majo), 54.9 (mino), 54.4 (majo), 31.7 (majo), 31.5 (mino), 31.1, 30.9 (majo), 30.4 (mino), 28.6 (majo), 28.5 (mino), 24.2 (majo), 23.8 (mino); MS (ESI): *m/z* 268 ([M+H]<sup>+</sup>); [α]<sub>D</sub><sup>20</sup> = -17.9 (c 1.0, MeOH).

Compound **11** (2 rotamers 55:45): <sup>1</sup>H NMR: (300 MHz, CDCl<sub>3</sub>) δ 5.70 (t, *J* = 5.2 Hz, 0.55H), 5.58 (t, *J* = 5.2 Hz, 0.45H), 4.50 (d, *J* = 7.7 Hz, 1H), 4.37 (m, 0.55H), 4.23

(m, 0.45H), 2.97–2.83 (m, 3H), 2.28–2.22 (m, 3H), 2.15–2.12 (m, 4H), 2.00–1.95 (m, 2H), 1.75–1.55 (m, 3H), 1.53–1.42 (m, 4H), 1.45 (s, 4.5H), 1.40 (s, 4.5H), 1.30–1.15 (m, 4H), 0.08 (s, 9H);  $^{13}\text{C}$  NMR: (75 MHz,  $\text{CDCl}_3$ )  $\delta$  171.7, 153.2 (mino), 152.8 (majo), 144.3, 143.9, 130.5 (majo), 128.4 (mino), 107.4, 84.4, 84.3, 79.4, 79.1, 77.4, 60.4, 57.3, 55.4, 51.0, 46.9, 37.6 (mino), 37.2 (majo), 32.6 (mino), 32.3 (majo), 31.4, 29.5, 28.5 (majo), 28.2 (mino), 26.8 (mino), 26.3 (majo), 23.5, 21.1 (mino), 19.8 (majo), 14.2, 0.2; IR ( $\text{CH}_2\text{Cl}_2$ ):  $\nu$  2932, 2860, 2173, 1693, 1621, 1404, 1364, 1248, 1172, 1110, 842  $\text{cm}^{-1}$ ; MS (ESI):  $m/z$  461 ( $[\text{M}+\text{H}]^+$ );  $[\alpha]_{\text{D}}^{20}$  +32.9 (c 1,  $\text{CHCl}_3$ ).

Compound **12** (TFA salt):  $^1\text{H}$  NMR: (300 MHz,  $\text{CDCl}_3$ )  $\delta$  9.81 (br s, 1H), 9.17 (br s, 1H), 6.12 (t,  $J = 5.2$  Hz, 1H), 4.43 (br s, 1H), 4.29 (br s, 1H), 3.40–3.20 (m, 2H), 3.05–2.82 (m, 3H), 2.57–2.41 (m, 3H), 2.39–2.25 (m, 1H), 2.18–2.12 (m, 4H), 2.00–1.88 (m, 2H), 1.85–1.73 (m, 1H), 1.55–1.44 (m, 4H), 1.41–1.33 (m, 2H), 1.31–1.14 (m, 2H);  $^{13}\text{C}$  NMR: (75 MHz,  $\text{CDCl}_3$ )  $\delta$  170.5, 161.4 (q, TFA), 137.5, 136.4, 84.5, 68.4, 59.0, 57.1, 47.8, 37.7, 31.6, 28.4, 28.3, 27.7, 26.9, 26.6, 26.3, 23.2, 18.3; IR ( $\text{CH}_2\text{Cl}_2$ ):  $\nu$  3411, 2943, 1694, 1674, 1651, 1615, 1455, 1206, 1138, 845, 800, 724  $\text{cm}^{-1}$ ; MS (ESI):  $m/z$  289.5 ( $[\text{M}+\text{H}]^+$ ); HRMS calcd for  $\text{C}_{18}\text{H}_{29}\text{N}_2\text{O}$ : 289.2280. Found: 289.2283;  $[\alpha]_{\text{D}}^{20}$  +24.7 (c 0.5,  $\text{CHCl}_3$ ).