52. Further Studies on the Biosynthesis of Tabtoxin (Wildfire Toxin): Incorporation of [2,3-$^{13}C_2$]Pyruvate into the β-Lactam Moiety

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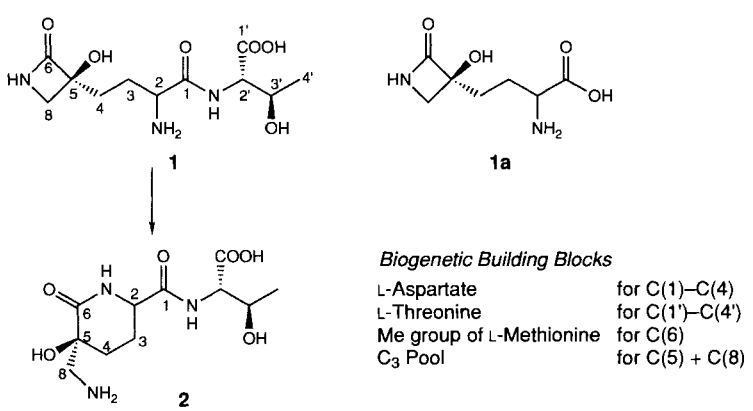
[2,3-$^{13}C_2$]Pyruvic acid (**7**) was synthesized and administered to cultures of *Pseudomonas syringae* pv *tabaci*. C(2) and C(3) of **7** were incorporated as an intact unit into the β-lactam moiety of tabtoxin (**1**). The result suggests that the biosynthesis of **1** is proceeding in part along the lysine pathway. The labelling pattern in **1** and an incorporation experiment with α,α'-dideuterated (±)-2,6-diaminopimelic acid (**19**) indicate that the branching in the biosynthesis of **1** occurs before lysine is formed.

Introduction. – Tabtoxin (Wildfire toxin; **1**) is an extracellular pretoxin produced by the phytopathogenic bacterium *Pseudomonas syringae* pv *tabaci*. The biologically active β-lactam **1** is unstable [1]. It is converted to the stable but biologically inactive δ-lactam isotabtoxin (**2**) by intramolecular transacylation. In our previous investigations on the biosynthesis of tabtoxin, three amino acids were found to be involved in forming the molecule [2]. Incorporation experiments with ^{13}C-labelled compounds demonstrated that L-aspartate and L-threonine are precursors of the side chain, whereas the Me group of L-methionine was found to provide the carbonyl C-atom of the β-lactam moiety. Furthermore, it was shown that the remaining C_2 unit of the β-lactam moiety is not derived from acetate as *e.g.* in thienamycin [3], but from an intermediate originating from the C_3 pool (*Scheme 1*). In this paper, we wish to report the synthesis and results of the incorporation experiments of doubly ^{13}C-labelled pyruvic acid **7** and α,α'-dideuterated diaminopimelic acid **18** (*cf. Scheme 4*).

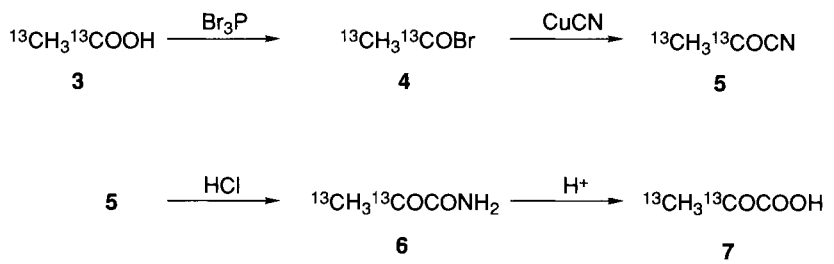
Scheme 1



Results and Discussion. – To improve the tabtoxin production, a suspension of *P. tabaci NCPPB 2730* was applied to its host, the tabacco plant, and re-isolated after *ca.* 5 days [2]. *Erlenmeyer* flasks containing *Woolley*'s medium [4] were inoculated and incubated on a rotary shaker at 28°. ^{13}C- and ^{2}H-Labelled potential precursors were administered by using pulse-feeding in 5 portions. The cultures were harvested after 72 h, yielding 50–100 mg/l isotabtoxin (**2**) as an artefact of the workup. Since the incorporation of the precursor into **2** is representative for the biosynthesis of **1**, no effort was made to isolate **1** in its original form (*Scheme 1*).

After the biogenetic origin of all C-atoms except C(5) and C(8) had been established, we assumed that the remaining C_2 unit in the β-lactam moiety was connected to the metabolism of the C_3 pool. This assumption is supported by the fact that racemic [1,2-$^{13}C_2$]glycerol is incorporated into this C_2 unit, whereas [1,2 -$^{13}C_2$]acetic acid is not [2]. To clarify this point, we decided to synthesize doubly labelled pyruvic acid for incorporation experiments. [2,3-$^{13}C_2$]Pyruvic acid (**7**) was prepared using [1,2-$^{13}C_2$]acetic acid (**3**) as starting material (*Scheme 2*). The latter was converted first to [2,3-$^{13}C_2$]acetyl bromide (**4**) [5] which reacted with CuCN to yield [2,3-$^{13}C_2$]pyruvonitrile (**5**) [6]. Addition of HCl gas led to [2,3-$^{13}C_2$]pyruvamide (**6**) from which the desired [2,3-$^{13}C_2$]pyruvic acid (**7**) was obtained by treatment with aq. HCl.

Scheme 2



The ^{13}C-NMR spectral lines of the enriched isotabtoxin (**2**) sample, isolated after incorporation of **7**, is shown in *Fig. 1*. The observed $^{13}C,^{13}C$-coupling pattern (*Fig. 2*) is consistent with the assumption that the atoms C(5) and C(8) are derived from pyruvate. A specific incorporation rate of 1.4% (*Table*) is found. The splitting pattern in the aspartate- and the threonine-derived part of the molecule is as expected. Pyruvate may enter the tricarboxylic-acid cycle in different ways. One of them leads from [2,3-$^{13}C_2$]pyruvate *via* [1,2-$^{13}C_2$]acetate into the cycle. Thus, [1,2-$^{13}C_2$]- or [3,4-$^{13}C_2$]aspartate is formed. In another route, **7** can be converted to [2,3-$^{13}C_2$]oxalacetate or to [2,3-$^{13}C_2$]malate. In this case, [2,3-$^{13}C_2$]aspartate is formed. The specific incorporation rate for C(2)/C(3) of the aspartate moiety of **1** is 1.0%. Since aspartate is a direct precursor of threonine, we find the same coupling pattern in **1** for C(1') to C(4') as for C(1) to C(4).

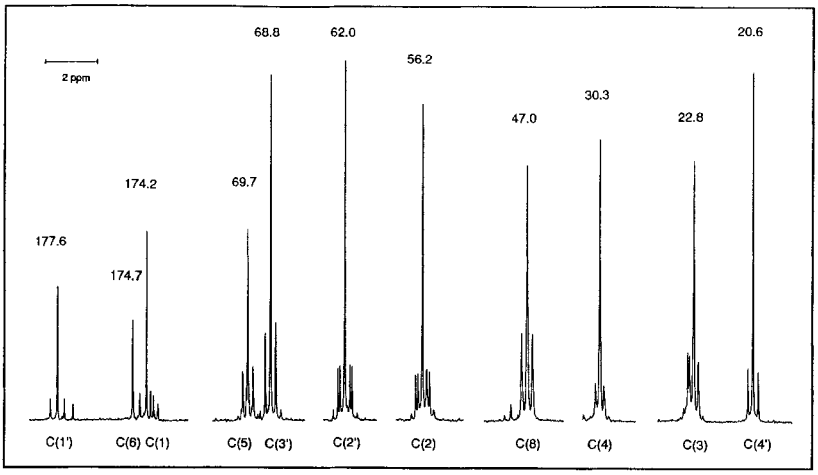


Fig. 1. *400-MHz Proton-noise-decoupled ^{13}C-NMR spectral lines* (D_2O) *of enriched isotabtoxin* (**2**) *after incorporation of [2,3-$^{13}C_2$]pyruvate.* From left to right: C(1'), C(6), C(1), C(5), C(3'), C(2'), C(2), C(8), C(4), C(3), C(4').

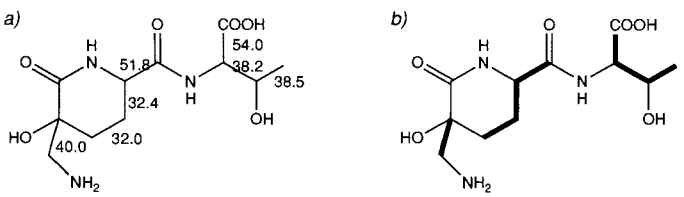


Fig. 2. a) *$^{13}C,^{13}C$-Coupling constants and* b) *coupling pattern in isotabtoxin after incorporation of [2,3-$^{13}C_2$]pyruvate*

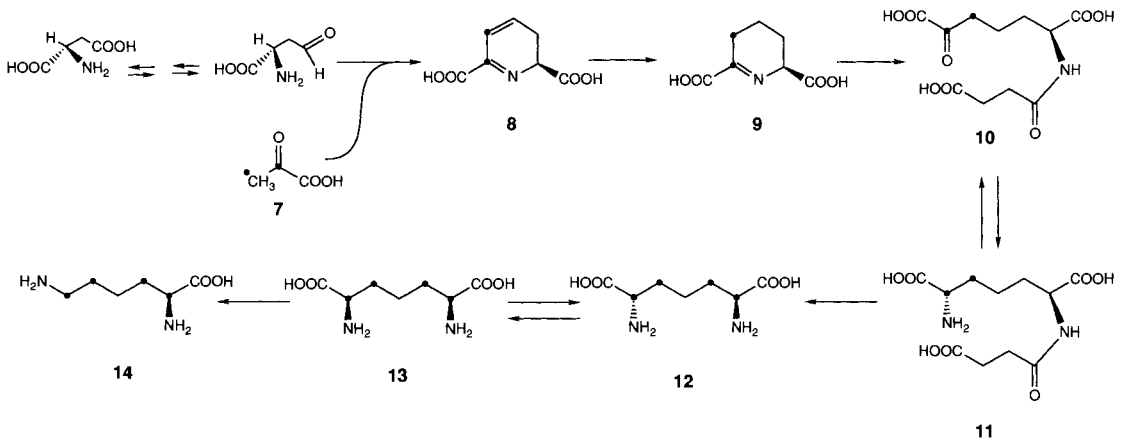
The comparison of the specific incorporation rates in this experiment with those obtained earlier after incorporation of [1,2-$^{13}C_2$]glycerol [2] clearly demonstrates that pyruvate is the more specific precursor (*Table*). *Unkefer et al.* [7] have recently reached the same conclusion, but on the basis of the incorporation of ^{13}C-labelled glucose.

Table. *Specific Incorporation Rates for Some C_2 Units of* **2** *after Incorporation of* rac-*[1,2 -$^{13}C_2$]Glycerol, [2,3-$^{13}C_2$]Pyruvate, [1,2-$^{13}C_2$]Acetate, and* L-*[1,2 -$^{13}C_2$]- and* L-*[3,4-$^{13}C_2$]Aspartic Acid* [8]

Precursor	Specific incorporation rate [%]			Source
	C(2)/C(3)	C(3)/C(4)	C(5)/C(8)	
rac-[1,2–$^{13}C_2$]Glycerol	1.1	1.3	0.77	[2]
[2,3 –$^{13}C_2$]Pyruvate	1.0	1.2	1.4	this work
[1,2-$^{13}C_2$]Acetate	2.6	3.5	0.0	[2]
L-[1,2-$^{13}C_2$]Aspartate/L-[3,4-$^{13}C_2$]Aspartate 1:1	2.6	2.9	0.0	[2]

Since the building blocks of the tabtoxinine moiety (*cf.* **1a**) of **1** are the same as in the biosynthesis of lysine (**14**; with exception of the Me group from methionine), the following working hypothesis can be postulated: the tabtoxin biosynthesis is identical with the lysine pathway [9] (*Scheme 3*), until an unknown intermediate is reached. A detailed analysis of the lysine pathway reveals six possible intermediates. Lysine itself may probably be precluded, because, in an earlier feeding experiment, [^{14}C]lysine was incorporated to a much lesser extent than [^{14}C]aspartate [2]. One of the six intermediates **8**–**13** shows a C_2 axis of symmetry. Thus, the labelling is distributed between two parts of the molecule (one molecule with the labelling in its left-hand side, one molecule with the labelling in its right-hand side). The corresponding compound is (*S*,*S*)-2,6-diaminopimelic acid ((*S*,*S*)-2,6-DAP; **12**).

Scheme 3

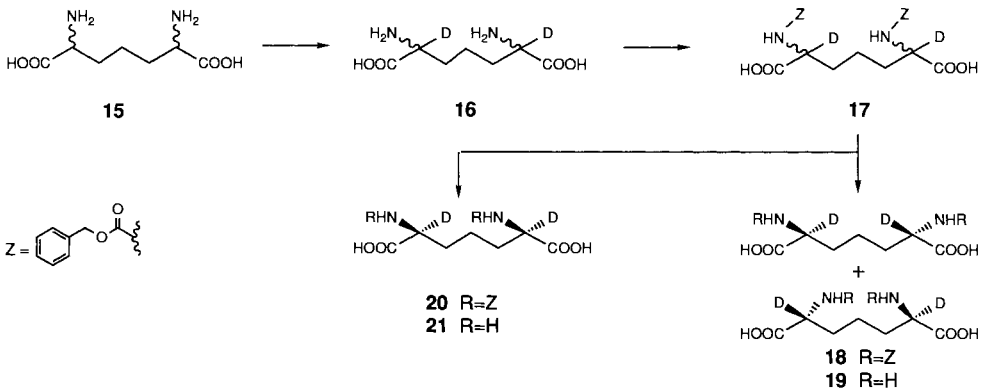


If our working hypothesis is correct, the incorporation experiment with **7** as well as the earlier experiments with doubly labelled aspartic acid, glycerol and AcOH [2] should provide further information. If the biosynthesis of tabtoxinine (**1a**) is branching off after the formation of **12**, then the distribution of labelled C_2 units in the chain C(2)–C(3)–C(4)–C(5)–C(8) of **2** should be symmetrical with respect to C(4). In other words, a coupling between the pairs C(3)/C(4) and C(4)/C(5) should appear at the same intensity. If we do not find this symmetry, we can assume that the branching is attained, before **12** is formed. This consideration is only valid on the assumption that **12** is not enzyme-bound, but is moving unhindered in the cytoplasm. Only in this case, free rotation around the C_2 axis with resulting distribution of the label is possible. With this restriction, our experiments indicate that the branching might occur before the formation of **12**, since we do not observe a symmetrical distribution of the label. In particular, a coupling between C(4) and C(5) has never been observed, whereas the C_2 unit C(3)–C(4) was labelled in all incorporation experiments mentioned above (*Table*).

Since the cyclic intermediates **8** and **9** on the lysine pathway are known to be unstable [10], they could not be synthesized and administered in a labelled form. We, therefore,

decided to perform incorporation experiments with (±)-[2,6-$^2H_2$]diaminopimelic acid ((±)-[2,6-$^2H_2$]DAP; **19**). For this purpose, 2,6-diaminopimelic acid (**15**) was deuterated by stirring with D_2O/KOH [11]. A mixture of diastereoisomers **16** is formed. To remove the *meso*-compound from the racemate, the isomers were converted into the benzyloxycarbonyl derivatives **17**, and the latter were separated by fractional crystallization (*cf. Wade et al.* [12]). Subsequent catalytic hydrogenolysis with Pd yielded pure (±)-[2,6-$^2H_2$]-DAP (**19**) and *meso*-[2,6-$^2H_2$]DAP (**21**).

Scheme 4



A sample of isotabtoxin (**2**) isolated, after administration of **19** was investigated by ^{2}H-NMR spectroscopy. With the technique used, an incorporation of > 0.25% would have been detected, but none was found at C(2) or at C(8). The stability of the C–^{2}H bond of the precursor under the usual pH conditions had already been tested. This result is consistent with the assumption that the biosynthesis of tabtoxin (**1**) is branching off from the lysine pathway before the formation of **12**. However, other reasons could as well explain the lack of incorporation of ^{2}H. Thus, **12** might not pass the bacterial cell membrane or might be metabolized in the primary metabolism prior to its possible use in tabtoxin biosynthesis.

The support of these investigations by the *Swiss National Science Foundation* is gratefully acknowledged.

Experimental Part

General. $^{13}CH_3{}^{13}COOH$ was purchased from *ICN Stable Isotopes*, Innerberg, Switzerland. *P. tabaci* strain *NCPPB 2730* was from the *National Collection of Plant Pathogenic Bacteria*, England. Stock cultures of *P. tabaci* were maintained on agar slants containing *NBY* medium [13] and stored at 4°. The tabacco plants (*Nicotiana Havanna*) were supplied by Prof. *F. Meins* (*Friedrich-Miescher Institute, Ciba-Geigy AG*, Basel). We thank Prof. *F. Meins* for this gift. The org. extracts were dried (Na_2SO_4) and evaporated under reduced pressure below 45°. TLC: silica gel *60* F_{254} (*Merck*); detection with UV or ninhydrin soln. Column chromatography: silica gel *60* (63–200 μm, *Merck*); cellulose microcristallin *Avicel pH 101* (50 μm, *Fluka*); solvent ratios in *v/v*. HPLC: *Nucleosil-*C_{18} (5 μm, 4.0 × 250 mm, *Macherey-Nagel*) with 0.1% CF_3COOH in H_2O or 0.1% CH_3COOH in H_2O. M.p.: *Kofler* Block, corrected. IR: *Perkin-Elmer-781* spectrometer. NMR:

Varian-EM-360 (^{1}H, 60 MHz), *Bruker-WH-90* with *Fourier* transform (^{1}H, 90 MHz; ^{13}C, 22.63 MHz), and *Varian VXR-400* spectrometer with *Fourier* transform (^{1}H, 400 MHz; ^{13}C, 101 MHz); chemical shifts in ppm relative to internal TMS or TSP (1.7 ppm). MS: *VG-70-250* instrument.

Production of Tabtoxin (= *(S)-N-[4-(3-Hydroxy-2-oxo-3-azetidinyl)-L-2-aminobutanoyl]-L-threonine*; **1**) *and Isolation of Isotabtoxin* (= *(2S-trans)-N-{[5-(Aminomethyl)-5-hydroxy-6-oxo-2-piperidinyl]carbonyl}-L-threonine*; **2**). Activation of bacteria on tabacco plants, growing of cultures, pulse feeding (150 mg/l of **7**, 250 mg/l of **18**), and isolation of **2** were carried out as described in [2]. ^{1}H-NMR (400 MHz, D_2O): 1.20 (*d*, *J* = 6.5, CH_3(4')); 1.96 (*m*, CH_2(4)); 2.07, 2.36 (*m*, *AB*, CH_2(3)); 3.13, 3.33 (*AB*, *J* = 13.5, CH_2(8)); 4.18 (*d*, *J* = 4.5, H–C(2')); 4.26 (*m*, H–C(3')); 4.36 (*t*, *J* = 11.0, H–C(2)). ^{13}C-NMR (101 MHz, D_2O): 20.6 (C(4')); 22.8 (C(3)); 30.3 (C(4)); 47.0 (C(8)); 56.2 (C(2)); 62.0 (C(2')); 68.8 C(3')); 69.7 (C(5)); 174.2 (C(1)); 174.7 (C(6)); 177.6 (C(1')).

[1,2-$^{13}C_2$]Acetyl Bromide (**4**) [5]. To 1.12 ml (11.8 mmol) of Br_3P, 1.9 ml (33 mmol) of [1,2-$^{13}C_2$]acetic acid (90% ^{13}C) were added under stirring within 10 min. After stirring at 40–50° for 1 h, distillation yielded 1.55 g (12.6 mmol) of **4**. B.p. 69–73°.

[2,3$^{13}C_2$]Pyruvonitrile (**5**) [6]. To 1.55 g (12.6 mmol) of **4**, 1.12 g (12.6 mmol) of CuCN were added within 20 min. After subsequent stirring at 80° for 2 h, 608 mg (8.8 mmol) of **5** were obtained by distillation. B.p. 86–90°. IR (CH_2Cl_2): 2200, 1728, 1360, 1170. ^{1}H-NMR (400 MHz, $CDCl_3$): 2.55 (*s*, CH_3).

[2,3-$^{13}C_2$]Pyruvamide (**6**) [6]. A soln. of 608 mg (8.8 mmol) of **5** in 35 ml of abs. Et_2O was saturated with dried HCl gas at 0°. After slow addition of 0.16 ml (8.8 mmol) of H_2O, the soln. was further treated with HCl gas for 10 min. The precipitate was washed with Et_2O/cyclohexane 5:1 and dried to yield 410 mg (4.7 mmol) of **6**. M.p. 120–123°. IR (CH_2Cl_2): 3510, 3390, 1710, 1570, 1350, 1170. ^{1}H-NMR (400 MHz, (D_6)DMSO): 2.32 (*s*, CH_3); 7.61 (NH); 7.86 (NH). ^{13}C-NMR (101 MHz, (D_6)DMSO): 24.6 (C(3)); 163.2 (C(1)); 197.7 (C(2)). EI-MS: 89 (M^+), 45 ([M – $^{13}CH_3CO$]$^+$, [M – $CH_3{}^{13}CO$]$^+$), 44 ([M – $^{13}CH_3{}^{13}CO$]$^+$).

[2,3 -$^{13}C_2$]Pyruvic Acid (**7**). A soln. of 410 mg (4.7 mmol) of **6** in 4.8 ml of 1N HCl and 5 ml of H_2O was refluxed for 2 h at 105°. After adding 1 g of NaCl, the product was extracted with Et_2O in a *Kutscher-Steudel* apparatus for 48 h. A subsequent bulb-to-bulb distillation (15 mm Hg, 110°) yielded 313 mg (3.5 mmol) of **7**. IR (CH_2Cl_2): 3400, 1785, 1730. ^{1}H-NMR (400 MHz, $CDCl_3$): 2.60 (*s*, $^{12}CH_3{}^{12}CO$; *d*, *J* = 130.0, $^{13}CH_3{}^{12}CO$; *d*, *J* = 7.0, $^{12}CH_3{}^{13}CO$; *dd*, *J* = 130.0, 7.0, $^{13}CH_3{}^{13}CO$); 8.68 (*s*, OH).^{13}C-NMR (101 MHz, $CDCl_3$): 24.7 (*s*,*d*; *J* = 45.0 C(3)); 159.9 (C(1)); 193.5 (*s*,*d*; *J* = 45.0 (C(2)). EI-MS: 90 (M^+), 45 (100).

2,6-Diamino[2,6-$^2H_2$]pimelic Acid (**16**) [11]. As described in [11], 2,6-diaminopimelic acid (**15**; a mixture of (*R*,*R*)-, (*S*,*S*)-, and (*R*,*S*)-isomers; 4.9 g, 25.7 mmol), KOH (5.85 g), and pyridoxal hydrochloride (525 mg; 2.57 mmol) were dissolved in 24.5 ml of D_2O. After stirring for 9.5 h, the solvent was removed by freeze-drying. The residue was dissolved in D_2O (35 ml), heated to reflux for 2 h, cooled, and the pH value adjusted to 5.0 by addition of 4N HCl. EtOH (100 ml) was added, until permanent cloudiness was observed. Cooling to 0° and filtration gave 3.47 g (21.2 mmol) of **16**. ^{1}H-NMR (400 MHz, D_2O): 1.48 (*m*, CH_2(4)); 1.91 (*m*, CH_2(3), CH_2(5)); 3.74 (*t*, *J* = 5.8, H–C(2), H–C(6) of (*R*,*S*)-isomer); 3.78 (*t*, *J* = 6.0, H–C(2), H–C(6) of (*R*,*R*)- and (*S*,*S*)-isomer, resp.). FAB-MS: 193 (100, [M + 3]$^+$, [2,6-$^2H_2$]DAP), 192 ([M + 2]$^+$, [2,6-$^2H_1$]DAP), 191 ([MH]$^+$, 2,6-DAP).

2,6-Bis[(benzyloxy)carbonylamino][2,6-$^2H_2$]pimelic Acid (**17**) [12]. As described in [12], 1.56 g (8 mmol) of **16** were dissolved in 20.5 ml of 1N NaOH. After dropwise addition of 3.2 ml (22.6 mmol) of benzyl chloroformate within 30 min at 0°, the mixture was stirred for 2 h at r.t. The soln. was extracted with AcOEt, then the aq. layer was acidified (4N HCl) to pH 1.7.

(±)-Bis[(benzyloxy)carbonylamino][2,6-$^2H_2$]pimelic Acid (**18**) [12]. The precipitated oil **17** was extracted with AcOEt, dried, evaporated to 8 ml, and stored at 4° for 8 days. The precipitated solid was recrystallized from AcOEt to yield 1.40 g (3.0 mmol) of **18**. M.p. 152–154°. ^{1}H-NMR (400 MHz, (D_6)acetone): 1.62 (*m*, CH_2(4)); 1.85 (*m*, CH_2(3), CH_2(5)); 4.23 (*m*, H–C(2), H–C(6), signals from undeuterated sites); 5.08 (*s*, CH_2(2'), CH_2(2'')); 6.5 (br., 2 COOH); 7.36 (*m*, 10 arom. H). FAB-MS: 461 [MH]$^+$, 460, 417, 91.

(±)-2,6-Diamino[2,6-$^2H_2$]pimelic Acid (**19**) [12]. According to [12] 1.39 g (3.0 mmol) of **18** were dissolved in 30 ml of AcOH/10 ml of H_2O and hydrogenated in presence of 70 mg Pd black. The catalyst was filtered off and the solvent evaporated *in vacuo*. The residue was taken up in 12 ml of H_2O and evaporated again. The residue was recrystallized from 35% EtOH to obtain 537 mg (3.0 mmol) of **19**. The material consisted of 30% of doubly-deuterated, 52% of single-deuterated, and 18% of undeuterated product. ^{1}H-NMR (400 MHz, D_2O): 1.47 (*m*, CH_2(4)); 1.91 (*m*, CH_2(3), CH_2(5)); 3.78 (*t*, *J* = 5.9, H–C(2), H–C(6)). FAB-MS: 193 ([M + 3]$^+$, [2,6-$^2H_2$]DAP), 192 (100, [M + 2]$^+$, [2,6-$^2H_1$]DAP), 191 ([MH]$^+$, 2,6-DAP).

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