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Structural studies of the teichoic acids from *Bacillus licheniformis*

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Abstract

Three teichoic acids have been isolated from *Bacillus licheniformis*. Their structures were found to be 1,3 poly(glycerolphosphate), → 3)-α-D-Gal*p*-(1 → 1)-L-glycerol-(3 → phosphate, and → 3)-[β-D-Glc*p*-(1 → 2)]-α-D-Gal*p*-(1 → 1)-L-glycerol-(3 → phosphate. Without separating the individual teichoic acids, and despite their high molecular weight, these polysaccharides could be identified by modern NMR techniques alone.

Keywords: Teichoic acids; $^{1}H-^{1}H$, $^{1}H-^{13}C$, $^{1}H-^{31}P$ 2D NMR

1. Introduction

Teichoic acids are major components of the cell walls of many Gram-positive bacteria, accounting for up to 50% of their dry weight. They include all wall-associated polymers, the repeating units of which are connected through phosphodiester linkages. There are several different types of teichoic acids and their structure, biosynthesis, and function have been reviewed [1–3]. We have previously identified three different teichoic acids in the fermentation supernatant of *Bacillus licheniformis*, a producer of thermostable amylase (Maxamyl)®, by NMR of their phosphodiester resonances.

The reproducibility of the fermentation of this overproducing strain is strongly dependent on correct phosphate levels in the broth. Classical phosphate analysis (phosphate after total destruction) showed high levels of phosphate under several different fermentation conditions [4]. Comparative analyses of the supernatants of these broths by means of NMR resulted in significantly different ^{31}P NMR spectra. It turned out that there was a build-up of three phosphodiester-containing compounds to such a level that in some fermentations the level of inorganic phosphate became limiting, after which production stopped. Increasing the level of inorganic phosphate and monitoring

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the level of inorganic phosphate instead of the total phosphate content solved the fermentation problems.

This paper reports the structure elucidation of the three teichoic acid in the mixture by means of the complete assignment of the ^{13}C, ^{1}H, and ^{31}P NMR spectra. Our general strategy for elucidating carbohydrate structures present in mixtures can be described as a modification of the strategy of van Halbeek [5] as follows. (*i*) Complete assignment of the ^{13}C NMR spectrum of the compound, mainly by {^{1}H–^{13}C} one bond correlation spectroscopy (HMQC) and a series of combined {^{1}H–^{13}C} HMQC–TOCSY experiments, assisted by {^{1}H–^{1}H} TOCSY experiments. (*ii*) Establishment of interglycosidic linkages by tracing long-range interglycosidic $^{3}J_{C,H}$ couplings by HMBC spectroscopy. (*iii*) Determination of the phosphodiester linkages by means of the earlier mentioned HMQC and HMQC–TOCSY experiments, but now {^{1}H–^{31}P}. This approach can be used succesfully on mixtures of polysaccharides, with molecular weights of up to 100,000.

2. Results and discussion

Bacillus licheniformis broth (48 h) was acidified with HCl to precipitate the extracellular protein, which was removed by centrifugation. The teichoic acids were isolated as boronate complexes essentially as described by Salt and Gander [6]. The ^{31}P NMR spectrum (see Fig. 3, vertical) of the teichoic acid mixture shows the three phosphodiester resonances. (Besides the three main components a number of minor components are visible in the ^{31}P and also in the ^{1}H NMR spectra; these minor components are probably acetylated teichoic acids, as in the ^{1}H NMR spectrum a number of acetyl groups are present at about the same level as the minor phosphodiester compounds.) Following acid-catalysed hydrolysis, galactose, glucose, and glycerol could be identified in the ^{1}H and ^{13}C NMR spectra in a ratio of about 3:2:4 and the ^{31}P NMR spectrum consisted of one single resonance of inorganic phosphate.

In the ^{1}H NMR spectrum (Fig. 1, top) there are three anomeric resonances at 5.24, 5.04 and 4.60 ppm (labelled A, B, and C) that can be used as a starting point in the analysis. From the ^{1}H and {^{1}H–^{13}C} HMQC spectrum (Fig. 1) it is clear that there is considerable overlap in the region 3.8–4.1 ppm and 69–70 ppm of the ^{1}H and ^{13}C spectrum. This makes the combined use of homo- and hetero-nuclear NMR spectra essential for the correct assignment of the individual resonances. {^{1}H–^{1}H} TOCSY (spectra not shown) and {^{1}H–^{13}C} HMQC–TOCSY spectra (Fig. 2) revealed that with long mixing times, the magnetization transfer in residue A and B was hampered at H-4, whose resonances also coincide for both residues. Given the small coupling constant $^{3}J_{H_4,H_5}$ in galactose (< 0.5 Hz), by which the magnetization will not be transferred to H-5, it can be concluded that A and B are Gal residues and C is the Glc residue, giving complete magnetization transfer at increasing mixing times through H-1 to H-6.

From the {^{1}H–^{1}H} TOCSY and {^{1}H–^{13}C} HMQC–TOCSY spectra most of the ^{1}H and ^{13}C chemical shifts can be extracted and they are summarized in Table 1. Besides the remaining Gal resonances (C/H-5 and C/H-6), the glycerol resonances and their interconnections cannot be easily extracted from these spectra. However, the presence of

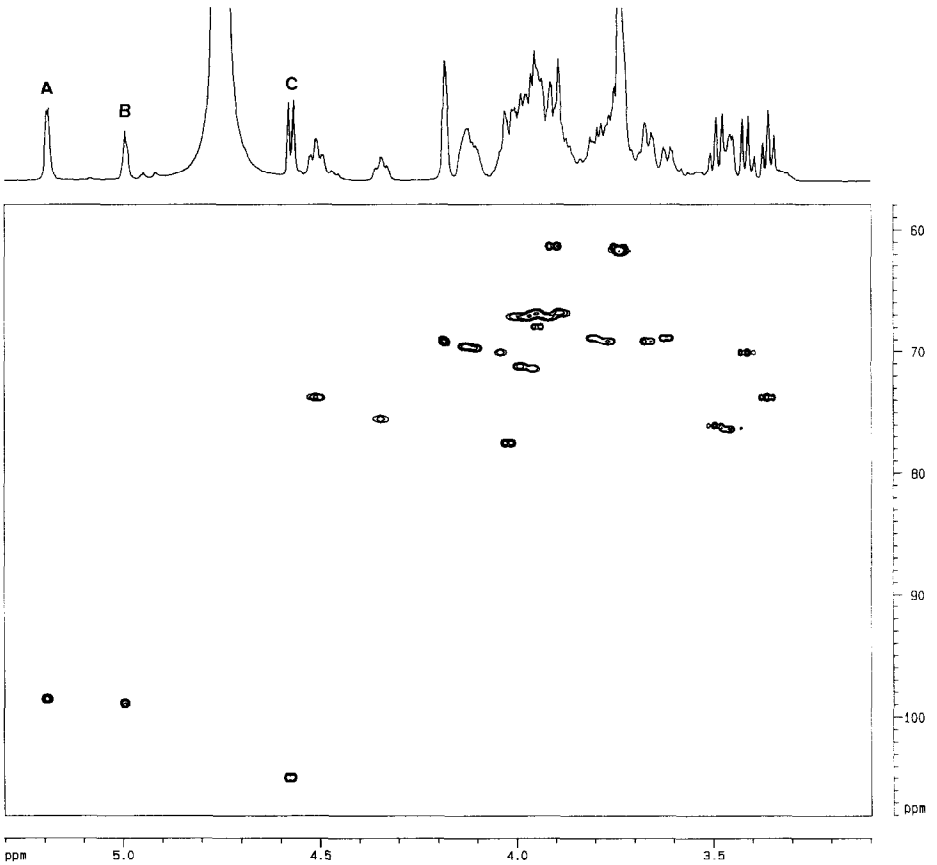

Fig. 1. {^{1}H–^{13}C} HMQC spectrum, with the high-resolution ^{1}H spectrum on top.

the phosphorus atom offers the possibility of performing the same HMQC and HMQC–TOCSY experiments as above, but with ^{31}P as the hetero atom. The {^{1}H–^{31}P} HMQC spectrum (Fig. 3) shows the three proton-detected phosphorus atoms. Two of the phosphorus atoms (II and III) are connected by an ester bond to the 3-position of the Gal residues at one site and a CH_2 group of glycerol at the other site. The diester bond of the third phosphorus is not clear at first sight but the {^{1}H–^{13}C} HMQC–TOCSY experiments (Fig. 2) do not show a connection to any of the Gal or Glc residues upon increasing the mixing time. Combining the {^{1}H–^{1}H} TOCSY, {^{1}H–^{13}C} HMQC–TOCSY, and {^{1}H–^{31}P} HMQC–TOCSY experiments, it can be concluded that the ^{1}H and ^{13}C chemical shifts of position 1 and 3 of this glycerol moiety are identical and that this compound must be a symmetric polymer of glycerol and phosphate, as has been found previously [3]. The magnetization transfer for the other two compounds in the {^{1}H–^{31}P} HMQC–TOCSY experiments shows the same Gal resonances as have been established by the {^{1}H–^{1}H} TOCSY and {^{1}H–^{13}C} HMQC–TOCSY experiments:

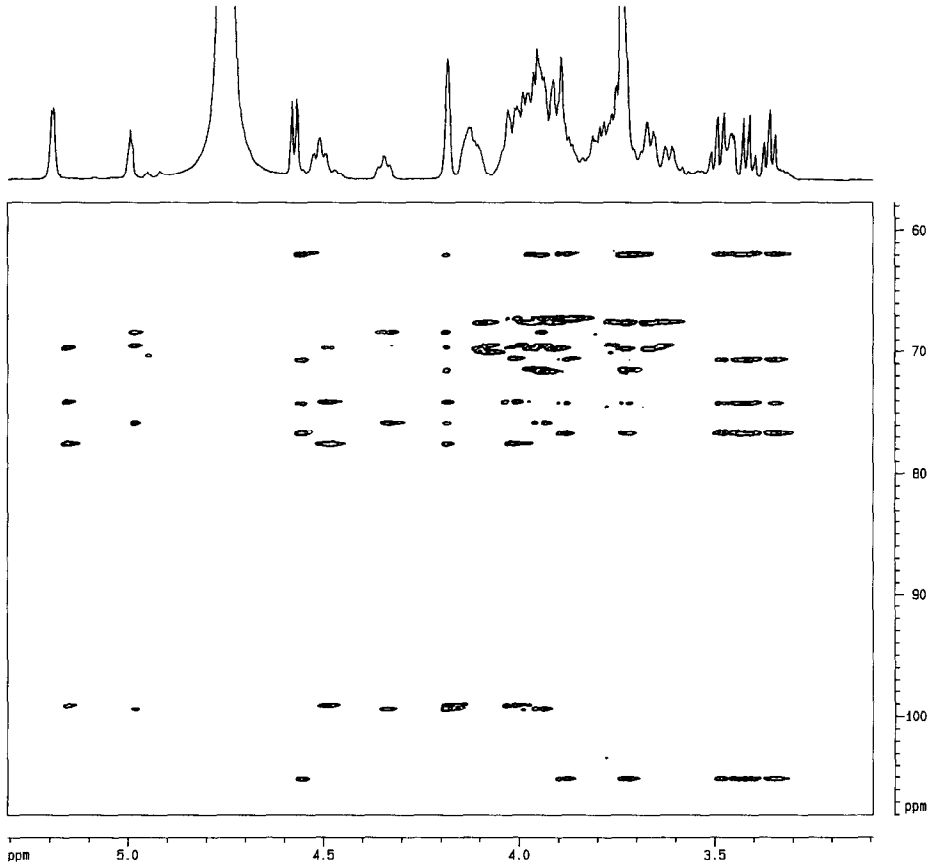

Fig. 2. {^{1}H–^{13}C} HMQC–TOCSY spectrum, with high-resolution ^{1}H spectrum on top. Mixing time 215 ms.

Table 1
^{1}H and ^{13}C chemical shifts of the constituent monosaccharides and reference chemical shifts for α-D galactose and β-D glucose [7]. The ^{13}C chemical shifts of the substituted carbon atoms are given in bold.

Atom	A	B	C	Gal α	Glc β
H-1	5.193	4.994	4.577	5.177	4.564
H-2	4.025	3.945	3.361	3.720	3.167
H-3	4.509	4.350	3.496	3.770	3.402
H-4	4.187	4.187	3.413	3.901	3.324
H-5	3.995	3.959	3.465	4.004	3.383
H-6′;H6″	3.738	3.738	3.904;3.741	3.643	3.814;3.641
C-1	**98.5**	**98.9**	**104.9**	92.6	96.2
C-2	**77.5**	67.9	73.7	68.7	74.5
C-3	**73.7**	**75.4**	76.0	69.5	76.1
C-4	69.0	69.0	70.1	69.7	69.9
C-5	71.2	71.3	76.4	70.8	76.2
C-6	61.6	61.6	61.3	61.5	61.1

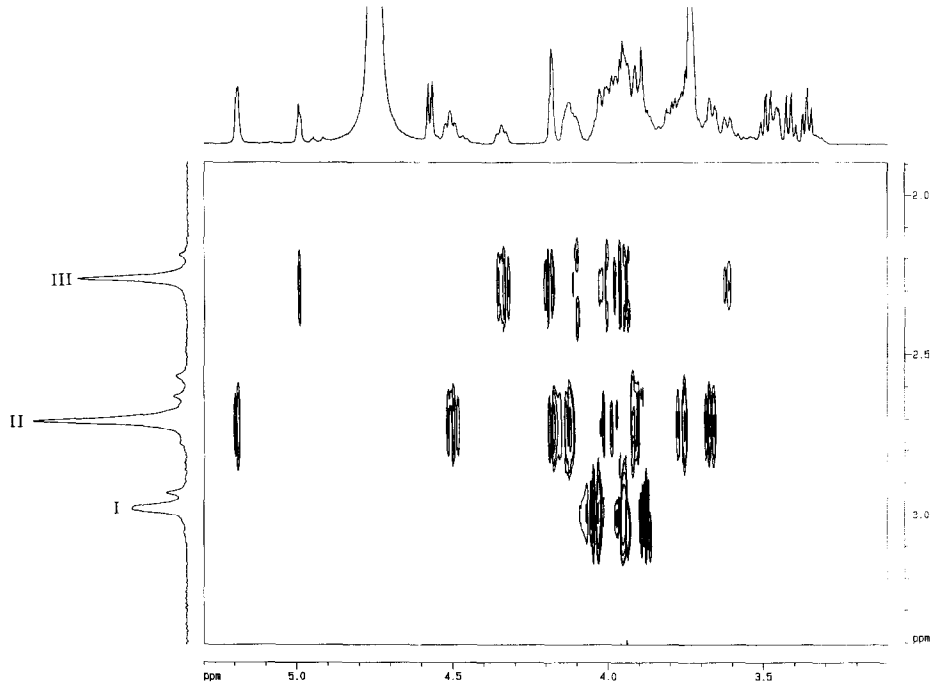

Fig. 3. {^{1}H–^{31}P} HMQC–TOCSY spectrum, with ^{1}H and ^{31}P spectra as projections. Mixing time 60 ms.

H-1–H-4. At the other site of the phosphodiester bond, magnetization is transferred from the two C1-protons via C2-H to the C-3 protons of glycerol.

The nature of the glycosidic bonds, and also the assignment of the remaining Gal residues, is most unequivocally determined by the ^{13}C HMBC experiment (Fig. 4). Despite the high molecular weight of the compounds (average 50 kDa), the HMBC spectrum gives very clear cross-peaks that make analysis quite straightforward. Gal A shows the expected intraresidual long-range coupling to C-3 and C-5, but also an interresidual coupling to C-3 of one of the glycerol moieties (indicated by A in Fig. 4). Gal B shows the same type of long-range couplings (B in Fig. 4), indicating that both Gal residues are linked to C-3 of glycerol that is linked to C-3 of the Gal residues via a phosphodiester bond at C-1. In addition, the position of the Glc residue became clear from the HMBC experiment: there is a strong cross-peak (C in Fig. 4) by the long range coupling of H-1 of glucose to C-2 of Gal A. From this experiment the missing chemical shifts can be extracted (Table 1). The substitution patterns of the monosaccharide units are completely supported by analysis of the ^{13}C chemical shifts (Table 1). Upon substitution, the ring carbon atoms show a downfield shift of about 5–10 ppm [8]. Resonances of C-1, C-2, and C-3 of Gal A show a significant downfield shift, as do the C-1 and C-3 resonances of Gal B. From the glucose unit only C-1 shows this downfield shift.

From the evidence presented the structure of all three teichoic acids (Fig. 5) could be established in the mixture by use of modern NMR techniques only.

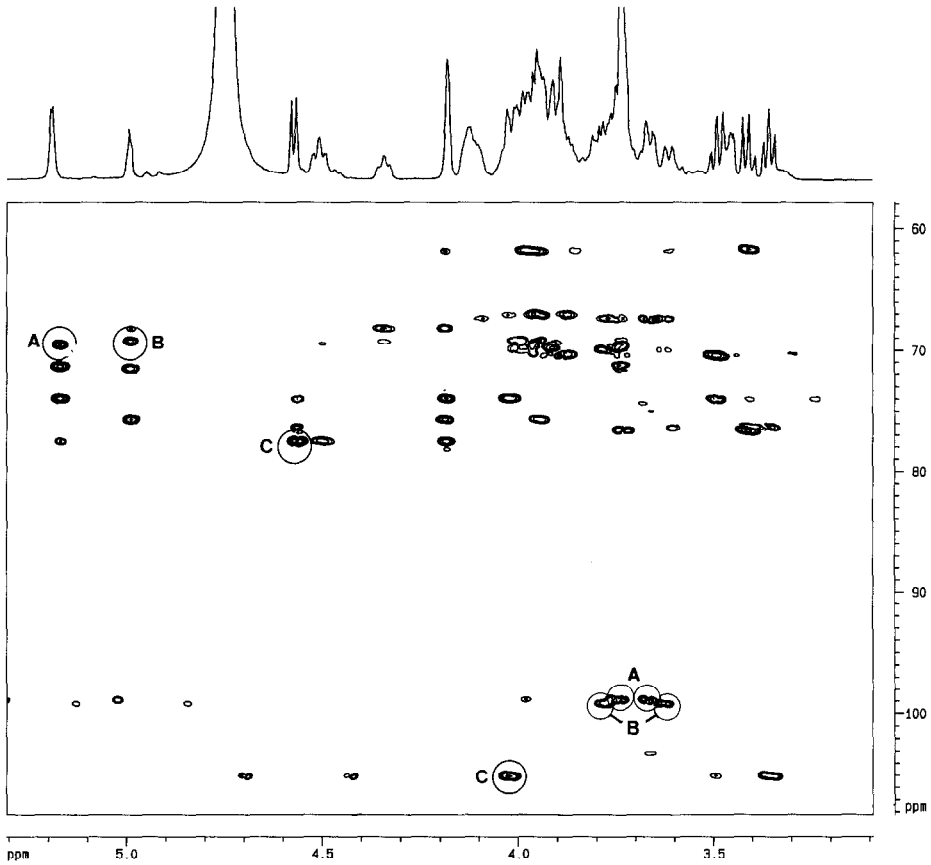

Fig. 4. {^{1}H–^{13}C} HMBC spectrum, with high-resolution ^{1}H spectrum on top. Indicated are the cross-peaks from the interglycosidic linkages between GalA C-1 and glycerol (A), between GalB C-1 and glycerol (B), and between Glc C-1 and GalB C-2 (C).

3. Experimental

Isolation of the teichoic acids.—The teichoic acids from *B. licheniformis* were isolated from the culture supernatant by precipitating the extracellular protein at pH 2.0 (HCl), centrifugation, and precipitating the crude polysaccharide in the supernatant by adding 1 volume of cold acetone. The precipitate was allowed to settle overnight at 4°C and then collected by centrifugation. Purification was achieved by isolating the teichoic acids as boronate complexes as described by Salt and Gander [6]. The yield was 25% on the basis of the crude polysaccharide.

NMR spectroscopy.—The NMR experiments were recorded on a Bruker AM-600 spectrometer at 25°C. Both the homonuclear and heteronuclear experiments were performed using a 5-mm broad-banded inverse probe on 50 mg polysaccharide in 0.5 mL D_2O. Chemical shifts were referenced as follows: ^{1}H to HDO (4.74 ppm), ^{13}C to

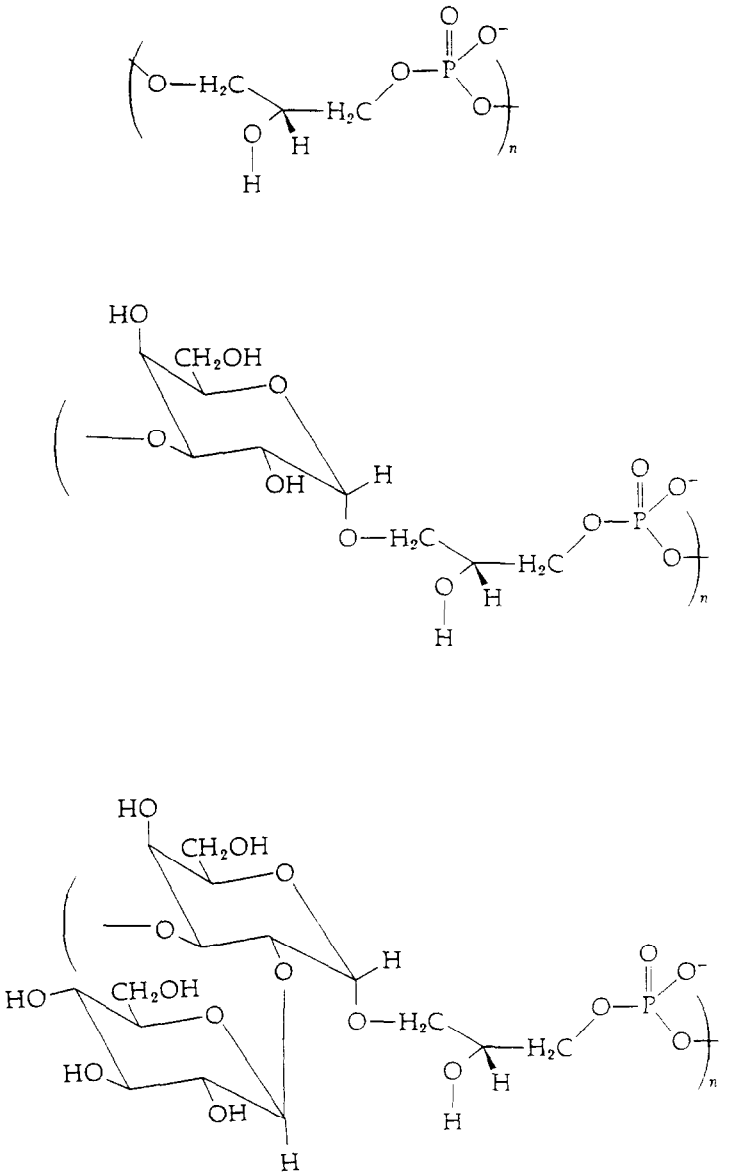

Fig. 5. Structures of the major teichoic acids of *Bacillus licheniformis*.

external dioxane in water (67.6 ppm), and ^{31}P to external 90% phosphoric acid (0 ppm). In each experiment the residual HDO signal was saturated during the 1.2 s recycle delay. TPPI was applied for phase-sensitive detection [9] (except for the HMBC experiment). The ^{1}H spectral width in all the spectra was 2400 Hz. The $\{^{1}H-^{1}H\}$ TOCSY experiments involved the clean-TOCSY sequence using the MLEV17 sequence [10] for

isotropic mixing; the delays during the mixing time were chosen equal to the 90° pulse width, which was 26 μs. The total spin-lock mixing time in the {^{1}H–^{1}H} TOCSY, as well as in the {^{1}H–^{13}C} HMQC–TOCSY experiment, ranged from 17 to 140 ms. A total of 256, 2K FIDs, 16 scans each, were collected, zero-filled once in the F2 dimension and twice in the F1 dimension. A squared sine-bell function shifted by $\pi/3$ was applied in both dimensions. The {^{1}H–^{13}C} HMQC [11] and {^{1}H–^{13}C} HMQC–TOCSY [12] experiments were preceded by a BIRD pulse [13]. The ^{13}C spectral width was 8600 Hz and ^{13}C decoupling during acquisition was achieved by the GARP-1 scheme [14]. A total 256, 2K FIDs of 32 scans were collected and the zero filling and digital filtering was identical to the {^{1}H–^{1}H} TOCSY experiments. The ^{13}C HMBC experiment [15] involved a low-pass filter (Δ_1 3.2 ms) and Δ_2 was chosen as 90 ms. A squared sine-bell function shifted by $\pi/12$ was applied in both dimensions.

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