Biosynthesis of Gabosines A, B, and C, Carba Sugars from *Streptomyces cellulosae*

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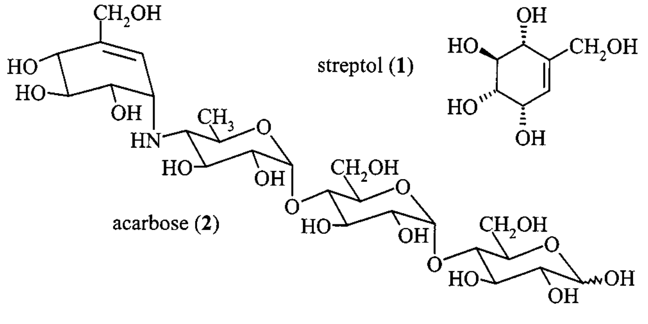
Keywords: Biosynthesis / Gabosine / Bioorganic chemistry / Carba sugars / *Streptomyces*

The biosynthesis of the gabosines A–C (**3**–**5**) was studied by feeding cultures of *Streptomyces cellulosae* subsp. *griseorubiginosus* (strain S 1096) with ^{13}C-labeled precursors. Although the carba sugars are structurally related to shikimic acid, the biosynthetic origin was found to be different to the shikimate pathway. The results revealed that the gabosines are formed via a pentose phosphate pathway by cyclization of a heptulose phosphate intermediate. This intermediate arises from a triose phosphate by successive transfer of two C_2 fragments by transketolases. This pathway is identical as that described for valienamine (**7**), the aminocarba sugar moiety of validamycin, and acarbose (**2**). The results from biosynthetic studies are discussed on the background of the variety of gabosines found in nature.

Introduction

Carba sugars and carba sugar containing secondary metabolites are widespread among microorganisms, especially actinomycetes. Besides the interest in the biological role of these compounds, they are discussed as synthetic building blocks for the preparation of more efficient drugs as substitutes for carbohydrate moieties.[1] In addition, some of the current interest in the synthesis of carba sugar containing molecules is stimulated by the broad spectrum of biological activities which has been reported.[2,3]

For example, the carba sugar streptol (**1**) is in use as a plant growth factor.[4] Salbostatin,[5] a trehalase inhibitor, like the agricultural antibiotic validamycin A,[6] is of interest as a pesticide. In addition, the aminocarba sugar valienamine (**7**), as part of validamycin A, is a constituent of the potent α-glucosidase inhibitor, acarbose (**2**) (Scheme 1). Compound **2** is a pseudo-tetrasaccharide used for the treatment of Diabetes mellitus.[7] The epoxycarba sugar cyclophellitol acts as a β-glucosidase inhibitor.[8] Carbocyclic analogues of nucleosides have been widely studied as effective antiviral, fungicidal, and anticancer agents.[9] Neplanocin A, for example, shows remarkable cytotoxic activity against different tumor cell lines.[10] The glyoxalase-I inhibitor COTC, a cytotoxic crotonic acid ester of the antibiotic KD16-U1,[11] has been isolated from the culture broth of *Streptomyces griseosporeus*,[12] and has been synthesized several times.[13,14]



Scheme 1. Structures of bioactive carba sugar containing secondary metabolites

Different biosynthetic pathways leading to carbocyclic C_7- and C_7N-units are known. The aminocarba sugars in acarbose **2**[15] or pyralomicin 1a[16] are biosynthesized by a pathway involving a C_7 sugar, which is formed from one C_3 and two C_2 units by a pentose phosphate pathway. Thus, carba sugar moieties are aliphatic analogues of aromatic *m*-C_7N units that are part of many antibiotics, e.g. the mitomycins,[17] pactamycin,[18] geldanamycin,[19] or the rifamycins.[20] Their *m*-C_7N unit is synthesized from a C_4 and a C_3 unit via a branch of the shikimate pathway. Similar fungal metabolites like epoformin[21] and terremutin[22] represent carba sugars formed by a polyketide assembly.

In addition to the examples of carba sugars being only a structural feature of a larger molecule, we discovered the ketocarba sugars called gabosines, which were found to be widespread in streptomycetes,[23] and could be detected by chemical screening methods only.[24] The gabosines are often produced in high yields (up to 410 mg/L), and differ structurally in both their substitution pattern and stereochemistry. The biological function of the gabosines is unknown, but they reflect a special metabolic pathway that allows for remarkable variety in the stereochemistry. Up to now, 15 gabosines are known, bearing a stereochemical variety similar to hexoses and deoxyhexoses.[23,25] This encouraged us to investigate the biosynthesis of the gabosines produced by one of our strains of *Streptomyces*, and to use these gabosines as an example of the biosynthesis of other gabosines. In this paper we report on the biosynthetic origin of all the carbon atoms of gabosine A (**3**), B (**4**), and C (**5**), see Scheme 2. This allowed us to propose a biosynthetic

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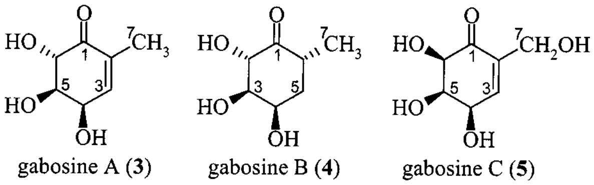


Scheme 2. Structure of selected gabosines

pathway for the formation of **3** to **5**, as well as the gabosine analogue **6**, a co-metabolite of *Streptomyces cellulosae* subsp. *griseorubiginosus* (strain S 1096).[23]

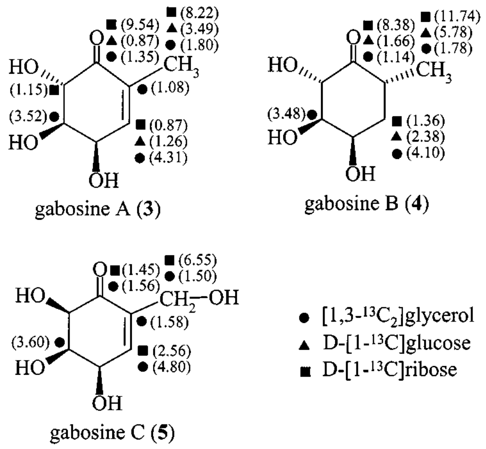
Results and Discussion

Fermentation and Isolation

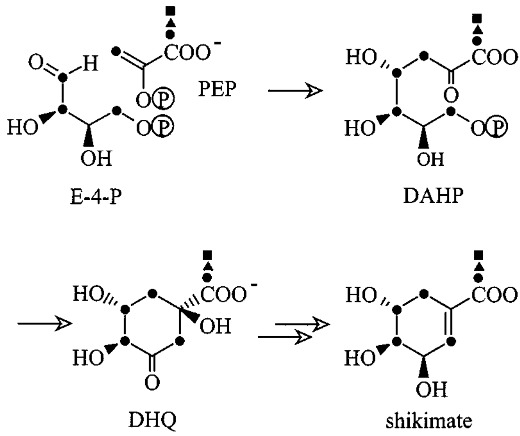
Batch fermentation of strain S 1096 was normally carried out in a 10-litre fermenter using a complex medium consisting of cooked rolled oats and a trace element solution. After 96 hours of fermentation 86 mg/L of gabosine A (**3**), 205 mg/L of gabosine B (**4**), 49 mg/L of gabosine C (**5**), and 15 mg/L of gabosine N (**6**) could be obtained by chromatographic purification.[25] For feeding experiments the strain was cultivated in shaking flasks. Production of gabosines A (**3**) and B (**4**) started after 24 hours, that of gabosine C (**5**) six hours later. The maximum yield was reached after 60 hours for **3** and 120 hours for **5**. Problems arise while planing the feeding experiments, due to these differences in the time scale. Since sufficiently high yields of gabosines were obtained after sixty hours, we decided to add the labeled precursors in pulse feeding experiments starting 32 hours after inoculation, and harvest the cultures after 63 hours, before the maximum of production of **5** is reached. A longer time between feeding and harvesting would promote scrambling of the precursors. Under these conditions, the yields of the gabosines A to C (**3** to **5**) were lower than in usual cultivations, (Table 1) and gabosine N (**6**) could not be obtained. In order to isolate the different gabosines, the culture filtrate was lyophilized and the crude product obtained was subjected to silica gel column chromatography. This was followed by gel permeation chromatography using Sephadex LH-20 with methanol or acetone as eluent.

Biosynthesis

Our first hypothesis for the biosynthesis of the carbon skeleton of the gabosines involved the structural relationship between the gabosines and shikimic acid. This led to the assumption that the carba sugars are built by the shikimate pathway. Feeding experiments with [1,3-$^{13}C_2$]glycerol, D-[1-^{13}C]glucose, and D-[1-^{13}C]ribose demonstrated a labeling distribution (Scheme 3) different from that expected for a shikimate derived metabolite (Scheme 4). The strong label of C-1 and C-3 (C-5 in **4**) besides that of C-7 after feeding of D-[1-^{13}C]ribose or D-[1-^{13}C]glucose is especially not compatible.



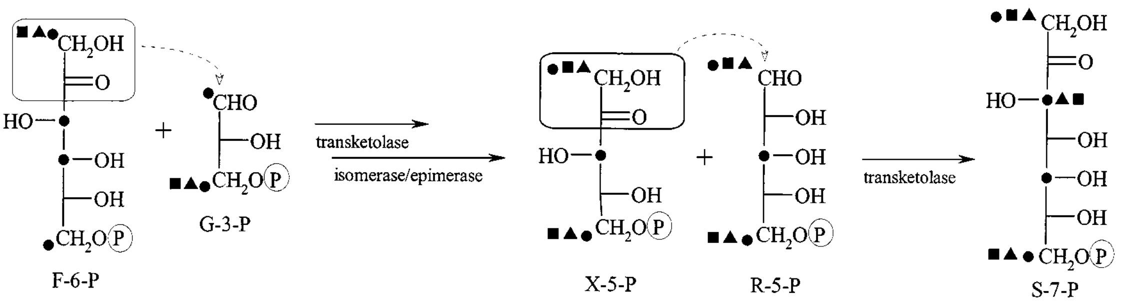
Scheme 3. Labeling of gabosines A to C (**3** to **5**) by different precursors. The data in parentheses show specific enrichments from individual precursors; relative enrichments were normalized to the peak intensity of C-4; enrichments for carbon atoms without data range from 0 to 0.2



Scheme 4. Conversion of phosphoenol pyruvate (PEP) and erythrose 4-phosphate (E-4-P) to shikimate (DAHP = 3-deoxy-D-*arabino*-heptulosonate-7-phosphate, DHQ = dehydroquinate); labeling pattern as shown in Scheme 3

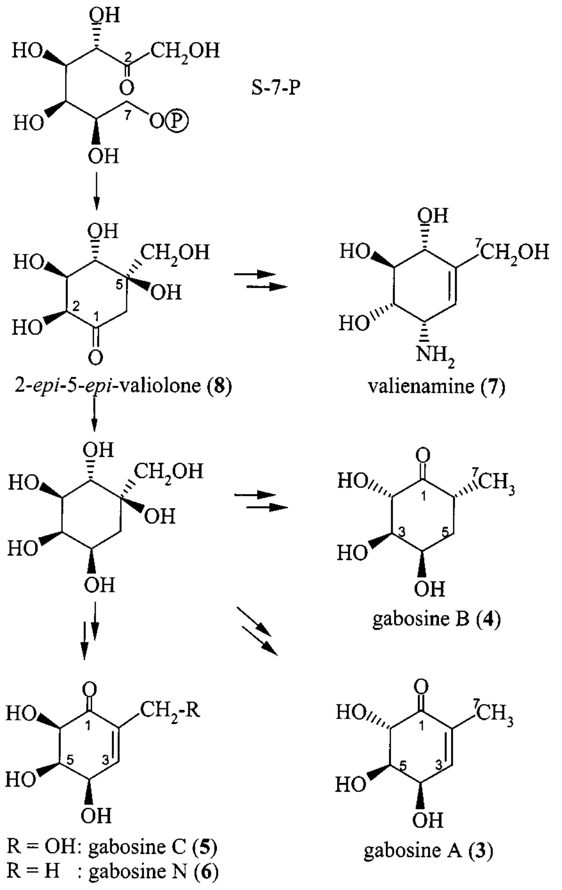
A second hypothesis pointed to a pentose phosphate pathway. The observed labeling pattern indicates that the gabosines are biosynthesized via a 7-carbon sugar intermediate (S-7-P), which originates from glyceraldehyde 3-phosphate (G-3-P) by transfer of a C_2 fragment from fructose 6-phosphate (F-6-P) by a transketolase (Scheme 5). The resulting xylulose 5-phosphate (X-5-P) can be converted into ribose 5-phosphate (R-5-P) by isomerisation and epimerisation. In the next step, a second C_2 fragment transfer from X-5-P to R-5-P takes place to form sedoheptulose 7-phosphate (S-7-P). The proposed cyclisation mechanism of S-7-P in the gabosine biosynthesis follows the known cyclisation pathway of DAHP for the construction of shikimate (Scheme 4 and 6).

Feeding experiments with [1,3-$^{13}C_2$]glycerol predominantly led to the labeling of C-1, C-3, C-5, and C-7 of the gabosines, which is consistent with the proposed biosynthetic pathway. Higher enrichments in C-3 and C-5 can be explained by the direct incorporation of glyceraldehyde 3-



Scheme 5. Biosynthetic pathway of sedoheptulose 7-phosphate (S-7-P) formed by iterative C_2 fragment transfer in a pentose phosphate pathway; labeling pattern as shown in Scheme 3

phosphate into this position. Additional experiments with [U-$^{13}C_3$]glycerol (data not shown) are not predicative. All the ^{13}C-signals showed ^{13}C-^{13}C couplings. Besides the coupling patterns resulting from intact incorporation of C_2 or C_3 segments of the precursor, statistical pairing of ^{13}C-labels caused ^{13}C-^{13}C couplings which do not allow a clear interpretation of the coupling pattern of all carbon atoms. However, the experiment revealed the intact incorporation of one glycerol unit into position C-3/C-4/C-5 of the gabosines. Feeding of D-[1-^{13}C]ribose resulted in strong enrichments of C-1 and C-7 of gabosine A (**3**) and B (**4**), and weaker labeling of C-3 in **3** and C-5 in **4**, respectively. As C-2/C-7 and C-1/C-6 of **3** as well as C-6/C-7 and C-1/C-2 of **4** arose directly from ribose 5-phosphate, this high incorporation could be easily explained. The additional weak label of C-1 of ribose found in C-3 of **3** (C-5 in **4**) corresponds to the interconversion of glyceraldehyde 3-phosphate by triose phosphate isomerase. Similar signal enhancements of C-1 and C-7 caused by D-[1-^{13}C]glucose also confirmed the proposed biosynthetic pathway.



Scheme 6. Proposed gabosine pathway starting from sedoheptulose 7-phosphate (S-7-P). The isolated gabosines are typical metabolites of strain S 1096

Conclusion

Based on the data of our feeding experiments, the biosynthesis of the gabosines obviously followed a pentose phosphate pathway in which sedoheptulose 7-phosphate (S-7-P) is a key intermediate. These findings correspond with results from biosynthetic studies on the valienamine (**7**) moiety in both validamycin A[26] and acarbose (**2**),[15] as well as on the cyclitol unit of pyralomicin 1a.[16]

In the case of the gabosines, we assume that sedoheptulose 7-phosphate cyclises by an aldol reaction between C-2 and C-7 to yield 2-*epi*-5-*epi*-valiolone (**8**) according to a recent proposal.[27,28] The stereochemistry of **8** at the newly formed center of chirality corresponds to that of DHQ, the cyclisation product of DAHP (Scheme 4). Compound **8** seems to be the starting point of all the known gabosines,[23,25] as well as of valienamine (**7**)[26] (Scheme 6). En route to the four isolated gabosines of the cultivated strain, a considerable number of steps such as dehydration, oxidation, reduction, and epimerisation are necessary. Concerning the sequence of the steps, a lot of questions remain open. The observation that gabosine C (**5**) appears very late in the fermentation broth could lead to the assumption that the deoxygenation of C-7 is an early step. It also seems possible that the reduction at C-7 and the oxidation at C-1 follow a similar mechanism, as described for the formation of 6-deoxy-4-keto sugars.[29,30] In this case, the formation of gabosine C (**5**) needs a renewed oxygenation at C-7.

Regarding both functional groups and stereochemical features, the gabosines isolated from different S*treptomyces* strains bear a large structural variety. However, a single strain produces a reproducible pattern of gabosines using stereochemically controlled biosynthetic transformations.[23,25] Remarkably, enantiomeric carba sugars could be isolated from different strains.

The gabosine pathway, branching off the pentose phosphate pathway via sedoheptulose 7-phosphate, seems to be one of the most flexible pathways compared with the size of the C_7 secondary metabolites. It can be seen as a mirror image of carbohydrate metabolism. Worldwide one can expect a considerably high number of specific working enzymes conserved in the genome of streptomycetes, and distributed on single strains in a manner that is really not understood.

Experimental Section

General: ^{1}H- and ^{13}C-NMR spectra were recorded with Varian VXR 200, Varian VXR 500, and Varian Inova 500 spectrometers using standard Varian software. − TLC was performed on silica gel plates (Merck, HPTLC ready-to-use plates, silica gel $60F_{254}$), and column chromatography on silica gel 60 (0.040×0.063, Merck) or Sephadex LH-20 (Pharmacia). − Fermentation was carried out in a Braun rotary shaker BS4 from Braun Dissel (Melsungen, Germany). − Soybean meal was obtained from Henselwerk GmbH, mannitol from Riedel-de Haën, and rolled oats from Koelln. All other chemicals were purchased from Riedel de Haën.

Labeled Isotopes: D-[1-^{13}C]glucose (99% ^{13}C) and D-[1-^{13}C]ribose (99% ^{13}C) were obtained from Cambridge Isotope Laboratories. [1,3-$^{13}C_2$]glycerol (49% ^{13}C) was synthesized from 2-bromoacetic acid and sodium [^{13}C]cyanide (99% ^{13}C) via [3-^{13}C]2-cyanoacetic acid, diethyl [1,3-$^{13}C_2$]malonate, and acetoxy [1,3-$^{13}C_2$]malonate.[31,32]

Culture Media. − Medium A: Soluble starch (1%), $(NH_4)_2SO_4$ (0.2%), $CaCO_3$ (0.2%), K_2HPO_4 (0.1%), NaCl (0.1%), $MgSO_4$ (0.1%), agar (2%), pH = 7.0 prior to sterilization. − **Medium B:** Soybean meal (degreased) (2%), mannitol (2%), pH = 7.2 prior to sterilization. − **Medium C:** Rolled oats (2%) (cooked for 20 minutes and filtered), 2.5 mL of a trace element solution of $CaCl_2 \times 2\ H_2O$ (3 g/L), Fe^{III} citrate (1 g/L), $MnSO_4$ (0.2 g/L), $ZnCl_2$ (0.1 g/L), $CuSO_4 \cdot 5\ H_2O$ (25 mg/L), $Na_2B_4O_7 \cdot 10\ H_2O$ (20 mg/L), $CoCl_2$ (4 mg/L), and $Na_2MoO_4 \cdot 2\ H_2O$ (10 mg/L).

Fermentation: A 1-cm^2 piece of agar from 7-d old cultures grown on medium A was used to inoculate 100 mL of medium B in 300-mL Erlenmeyer flasks. These cultures were cultivated on a rotary shaker (180 rpm) at 30 °C for 3 d. A 1-mL portion of this culture was used to inoculate 100 mL of medium C in a 300-mL Erlenmeyer flask. The cultures were harvested after 63 h.

Table 1. Conditions and yields of the gabosines after feeding [1,3-$^{13}C_2$]glycerol (I), D-[1-^{13}C]glucose (II), and D-[1-^{13}C]ribose (III)

	I	II	III
Precursor [g/L]	1	1	1
Culture volume [mL]	500	200	200
Yield [mg]			
3	23	5	10
4	54	1	11
5	2	-	5

Feeding Experiments: Labeled precursor aliquots (Table 1) were dissolved in sterile water and adjusted to pH 7. Feedings were carried out by the addition of labeled precursors in five equal aliquots following the pulse feeding method 32, 36, 40, 44, and 48 hours after incubation. Specific incorporation rates are summarized in Scheme 3 (calculation according Scott et al.[33]).

Isolation and Purification of Gabosines: The culture broths were separated from the mycelia by centrifugation (4500 rpm, 10 min), and the culture filtrates were concentrated by lyophilization. The crude products were extracted three times with methanol, the combined organic layers evaporated to dryness and the remaining residue was purified by chromatography on silica gel (4 × 30 cm, chloroform/methanol, 7:3) to afford two fractions. Gabosine A (**3**) and B (**4**) could be obtained in different yields (Table 1) by chromatography of the first fraction on Sephadex LH-20 (3 × 80 cm, acetone). The second fraction was purified by chromatography on Sephadex LH-20 (3 × 90 cm, methanol) to give gabosine C (**5**) in yields as shown in Table 1.

Characterization of Gabosines: The isolated gabosines A to C (**3−5**) were identified by their R_f-values (silica gel, chloroform/methanol, 9:1 and 7:3),[23] ^{1}H-NMR data[23] and ^{13}C-NMR data see Table 2. The assignments were done unambiguously by 2D-NMR measurements.

Table 2. ^{13}C NMR signals of gabosines A to C (**3** to **5**) at 50.3 MHz in CD_3OD

Gabosine	C-1	C-2	C-3	C-4	C-5	C-6	C-7
A (**3**)	200.4	136.9	143.0	67.4	73.9	75.1	15.6
B (**4**)	211.4	78.2	79.2	69.8	38.1	37.9	13.8
C (**5**)	199.3	137.8	145.0	69.4	77.7	76.9	59.3

Acknowledgments

We express our thanks to Hoechst AG (Frankfurt) for providing us with *Streptomyces cellulosae* (strain S 1096) and Matthias Decke for excellent technical assistance. This work was supported by the Fonds der Chemischen Industrie.

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Received October 22, 1999
[O99589]