COMMUNICATIONS TO THE EDITOR

Biosynthesis of (2*R*)-4-Amino-2-hydroxybutyric Acid, Unique and Biologically Significant Substituent in Butirosins

Sir:

Aminoglycoside antibiotics containing 2-deoxystreptamine as central aglycon constitute a major class of clinically important antibiotics. Among the typical members of this class including neomycins, kanamycins, gentamicins, ribostamycin, *etc.*,[1~3] butirosins are distinct from these members in two aspects, *i.e.* one is that producing organisms of butirosins belong to *Bacillus* sp. rather than *Actiomycetes* for others, and the other is that the (2*R*)-4-amino-2-hydroxybutyryl substituent is additionally attached to the C-1 amino group of the 2-deoxystreptamine aglycon in butirosins. The (2*R*)-4-amino-2-hydroxybutyric acid (AHBA) moiety is particularly significant in exerting antibacterial activities against resistant bacteria, and this medicinal advantage is in fact incorporated on the commercial bases into the modified antibiotics as amikacin and arbekacin.[4] In spite of such medicinal significance, little is known for the biosynthesis of the AHBA moiety.[5] Most of the biosynthetic studies in this class of antibiotics have been devoted to elucidating the origins and mechanistic aspects of the biosynthesis of 2-deoxystreptamine and other carbohydrate moieties as well as their assembling pathways.

Since we have witnessed rapid progress of genetic analysis and engineering of antibiotic biosynthesis, it appears to be significant at this point to clarify the biosynthetic pathway of AHBA, which may turn out to be beneficial in the future to develop diversities of this class of antibiotics.

The biosynthetic precursor of AHBA can be envisioned to be a rather simple metabolite based on its chemical structure. Previously, it was noted that supplementation of some amino acids showed improvement of the production of butirosin antibiotics to some extent, but such observation appeared not to be related for the biosynthesis of AHBA.[6] In the present study, we took advantage of isotope-tracer technology, particularly with ^{2}H- and ^{13}C-stable isotopes, to elucidate the biosynthetic pathway of AHBA in butirosin-producing *Bacillus circulans* SANK72073.

Based on its chemical structure, we anticipated that AHBA is derived from rather simple amino acid and that the hydroxy group at C-2 may be introduced at a later stage of biosynthesis. Glutamate and ornithine came to our mind as a possible precursor since decarboxylation of these amino acids would give this type of 4-aminobutyric acid (GABA) structure or an amino aldehyde equivalent *via* putrescine, respectively.

Labeling experiments with deuterated glutamic acid and ornithine were first attempted. Racemic [2,3,3-$^{2}H_3$]glutamic acid (*ca.* 90 atom% enriched at C-3 and *ca.* 30 atom% enriched at C-2) was prepared according to the previously described procedure.[7] L-[5,5-$^{2}H_2$]Ornithine (*ca.* 99 atom% enriched) was synthesized by a method adopted from a literature protocol.[8] Supplemented culture of *B. circulans* SANK72073 was carried out in a media (pH 7.5) consisting of 1.0% glycerol, 1.0% beef extract, 1.0% polypeptone, and 0.5% NaCl in distilled water, at 37°C for 5 days. The deuterated amino acids were separately supplemented in a pulse-feeding manner to the culture after 24, 36, and 48 hours from inoculation, and the feeding amount were 3 g/liter of glutamate or 0.4 g/liter of ornithine each time. Biosynthesized butirosin was subsequently isolated and purified according to the literature procedure.[9] The obtained butirosin in this manner was analyzed in ^{2}H-NMR as shown in Fig. 2. Deuterium incorporation into C-3‴ of the AHBA moiety was clearly observed in the case of deuterated glutamate feeding (Fig. 2A), but no deuterium was incorporated from deuterated ornithine (data not shown). The finding that incorporation of C-2 deuterium of glutamate was not observed at this time may be ascribable either to rather insufficient enrichment of the starting substrate or to involvement of a particular pathway in which the C-2 hydrogen of glutamate can be exchanged. In any

Fig. 1. Structure of butirosins.

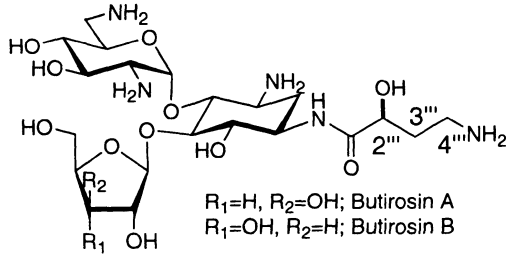
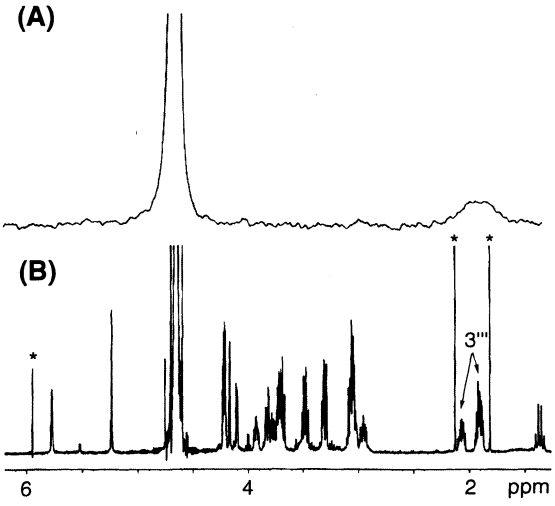
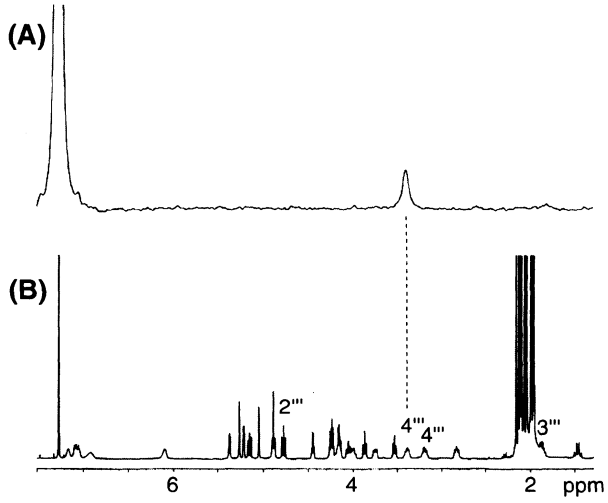


Fig. 2. ^{1}H and ^{2}H NMR spectra of butirosin.



(A); ^{2}H NMR spectra (77 MHz, H_2O) of butirosin produced by feeding with DL-[2,3,3-$^{2}H_3$]glutamic acid.
(B); ^{1}H NMR spectra (500 MHz, D_2O) of non-labeled butirosin.
Asterisk denotes signals due to impurities.

Fig. 3. ^{1}H and ^{2}H NMR spectra of peracetylated butirosin.



(A); ^{2}H NMR spectra (77 MHz, $CHCl_3$) of peracetylated butirosin obtained through feeding with [4,4-$^{2}H_2$]GABA.
(B); ^{1}H NMR spectra (500 MHz, $CDCl_3$) of non-labeled peracetylated butirosin.

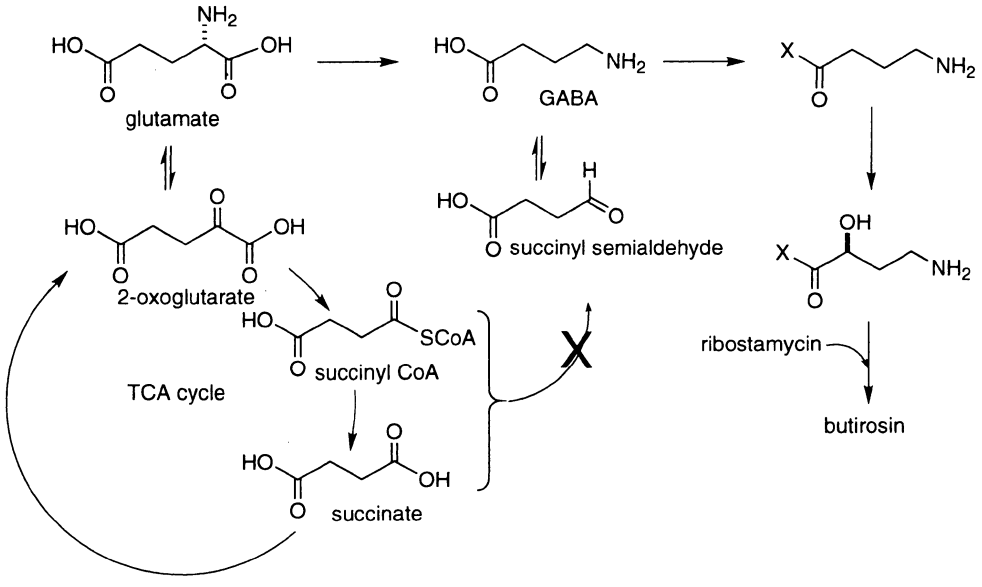
case, glutamate was proved to be a precursor to AHBA. In order to figure out more advanced intermediates, possibility of involvement of GABA and AHBA itself was next examined.

Necessary substrates were synthesized again by the procedures adopted from the literatures. [4-$^{2}H_2$]GABA (*ca.* 99 atom% enriched) was prepared according to the method in the literature[10] *via* reduction of succinimide with $LiAl^{2}H_4$. DL-[2-^{2}H]AHBA (99 atom% enriched) was prepared through protection of commercially available non-labeled AHBA as *N*-Boc methyl ester derivative,[11] oxidation with Dess-Martin reagent, reduction with $NaB^{2}H_4$, and final deprotection with HCl. Feeding experiments were carried out as described above, and 1 g/liter each of the labeled GABA and AHBA was separately supplemented to the culture with the same time schedule. Each resulting butirosin specimen was spurified and was further acetylated conventionally so that signal separation and assignment of ^{1}H- and ^{2}H-NMR spectra were fully pursued.

As can be seen in the ^{2}H-NMR spectra of the acetylated butirosin, a clear signal was observed for the labeled sample with GABA (Fig. 3A), but no incorporation of deuterium was observed for the AHBA feeding (data not shown). Apparently, GABA is an intermediate in the biosynthesis of AHBA, however, the free form of AHBA is not a competent intermediate. Hydroxylation at C-2 of GABA may thus be taken place at the later stage of the butirosin biosynthesis, or only certain activated form of GABA may be susceptible to such hydroxylation leading to AHBA. Worth and interesting to note here is that only one deuterium of [4,4-$^{2}H_2$]GABA was incorporated stereospecifically into C-4''', although the cryptic stereochemistry has yet to be elucidated. This observation strongly suggests involvement of rapid interconversion between GABA and succinyl semialdehyde.

Further, in order to test whether or not succinate in the TCA cycle is transformed into GABA, labeling experiments with commercially available [2,2,3,3-$^{2}H_4$]succinic acid and sodium [1-^{13}C]acetate were also carried out. Succinate incorporation was chased similarly by pulse-labeling (1.0 g/liter each), and the resulting butirosin was analyzed again by ^{2}H NMR after peracetylation. Acetate incorporation was examined by ^{13}C-NMR with purified butirosin without derivatization. While a modest ^{13}C enrichment (*ca.* 2%) was observed at C-1''' of the AHBA moiety in the [1-^{13}C]acetate labeling, no enrichment was observed at C-4''' at all. Further, no deuterium was incorporated into butirosin from

Fig. 4. Proposed biosynthetic pathway of AHBA moiety in butirosin.



[$^2H_4$]succinate (data not shown). These results appeared to suggest that the active TCA cycle gives rise to labeled glutamic acid from acetate, but a hypothetical pathway from succinate to succinate semialdehyde is not operative in the biosynthesis of the AHBA moiety of butirosins.

The overall pathway of the formation of AHBA moiety in butirosin elucidated in the present study is now illustrated in Fig. 4. It should be pointed out here that we suggested, based on the sequence homology, three possible genes for the AHBA biosynthesis in the butirosin biosynthetic gene cluster, that is, *btrK* for a PLP-dependent decarboxylase, *btrO* for a monooxygenase, and *btrI* for an acyl carrier protein.[12] Further genetic and enzymatic analysis should shed light on this small but interesting biosynthetic problems.

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