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THE BIOSYNTHESIS OF ANTIBIOTIC A23187

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Abstract- The biosynthesis of antibiotic A23187 (1) by *S. chartreusis* was examined using ^{14}C and ^{13}C labeled substrates in conjunction with chemical degradation and ^{13}C NMR. It has been demonstrated that the three distinct structural units of the antibiotic are derived from proline (the α-ketopyrrole unit), propionate acetate (dioxaspiro(5,5)undecane ring), glucose metabolism via a shikimate-type pathway (C_7N; or diaminobenzyl unit), and the S-methyl group of methionine (methyl amino of the C_7N; unit). Unlike antibiotics that contain a C_7N; unit, shikimic acid [^{14}C] was incorporated efficiently into the antibiotic and apparently specifically into the C_7N; unit. The incorporation of D-glucose[1-^{13}C], [6-^{13}C] and [U-$^{13}C_6$] is also consistent with a shikimate-type pathway to this unit. None of the aromatic compounds tested were incorporated into the C_7N; unit, suggesting that the introduction of the nitrogen functionalities in this moiety occurs before or concommitently with aromization of the six membered carbocyclic ring. These results are in accord with the involvement of a unique biosynthetic pathway in the formation of A23187.

Antibiotic A23187 (1) is a divalent cation ionophore produced in shake culture by *Streptomyces chartreusis* (NRRL 3882)[1]. This antibiotic is active against gram-positive bacteria and fungi and has been extensively used to study the biochemical role that divalent cations have in regulating cellular physiological processes.[2] A23187 apparently functions by breaking down cell membrane permeability barriers to divalent cations resulting in a variety of cell responses.[3] Being a small molecule and having only a single carboxylic acid, two molecules of the antibiotic are apparently required to carry out cation translocation processes.[4] A23187 is also the first polyether ionophore to use N-liganding groups which is undoubtedly responsible for its specificity for divalent cations.[5]

The structure of A23187 (1) indicates that it is a member of a new class of ionophorous antibiotics, the pyrrole polyethers. The only other member of this class is the recently discovered X-14547A.[6] Antibiotic A23187 is a monocarboxylic acid consisting of three distinct structural units, an α-ketopyrrole, a dioxaspiro(5,5)undecane ring and a C_7N; unit which forms the aromatic ring of the larger benzoxazole moiety of the antibiotic. Recently, the total synthesis of A23187 has been carried out and the absolute configuration of A23187 was determined to be correct.[7] The unusual structure of this antibiotic suggests an equally unique biosynthesis. The α-ketopyrrole could be derived from proline, δ-aminolevulinic acid or direct condensation of acetate and/or propionate units. Based on the known biosynthesis of macrolide antibiotics which lack an aromatic center[8] and antibiotics containing an aromatic center,[9] it would appear reasonable to assume that the spiroketal ring of A23187 is derived from acetate and propionate units. Another possibility is that the ring arises by condensation of acetate units with the Me groups arising through transmethylation from a C_1 donor, as has been demonstrated in the biosynthesis of aurodox[10] and boromycin.[11] The C_7N; unit having two nitrogens *ortho* to the exocyclic carbon is a structural unit unique to A23187. This unit could arise from four acetate units as described for salicylic acid or acetylphloroglucinol,[12] or from anthranilic acid, 3-hydroxyanthranilic acid via tryptophan[11] or glucose metabolism via a shikimate-type pathway. A number of interesting antibiotics contain a C_7N; unit having one nitrogen *meta* to the exocyclic carbon.[14] The biosynthetic origin of these units has been demonstrated to be via a divergence of the shikimate pathway prior to the formation of shikimic acid. In this paper, we report results on the biosynthesis of A23187 which demonstrate that the biosynthetic precursors for the antibiotic are proline, propionate, acetate, methionine and glucose via shikimic acid.[15]

RESULTS

The biosynthetic origin of the carbon skeleton of A23187 was examined using ^{14}C-labeled substrates to first distinguish between the several possible pathways already mentioned. Based on time course studies with the producing organism in shake culture, the labeled substrates were added to antibiotic producing cultures at 72 hr of cell growth. Cultures (50 ml of producing media) were harvested 24 hr later and A23187 was isolated as its calcium magnesium salt, converted to its acid form with HCl, methylated with diazomethane and purified twice by chromatography.[15] The antibiotic producing culture consistently yielded between 5 and 8 mg per 50 ml culture. The results, summarized in Table 1, indicate that propionate, acetate, proline, methionine, glucose and shikimic acid are efficiently incorporated into the antibiotic.

In order to obtain some information on the labeling pattern of these substrates, degradation of the specifically labeled A23187 methyl esters was carried out. Kuhn-Roth oxidation[16] of the antibiotic allows C-19' and -19,-17' and -17,-15' and -15,-11' and -11 to be removed from the molecule in the form of sodium acetate. Schmidt degradation[16] of this sodium acetate yields monomethylamine and CO_2.

Table 1 Incorporation of radioisotope labeled substrates into A23187 by *S. Chartreusis*

Exp.	Substrate	Quantity fed (umole)	Radio-activity fed ($dpm \times 10^6$)	Radio-activity in extract (% of total fed)	%incorp. of Substrate into A23187 Methyl ester
1.	L-Proline (U-^{14}C)	0.02	13.8	26.1	24.2
2.	D,L Glutamate (1-^{14}C)	0.19	19.4	1.4	<0.1
3.	D,L-Glutamine (5-^{14}C)	0.21	22.0	3.7	<0.1
4.	δ-Aminolevulinate (4-^{14}C)	0.20	22.0	7.2	<0.1
5.	Sodium Acetate (1-^{14}C)	6.63	34.9	4.4	1.8
6.	Sodium Acetate (2-^{14}C)	0.45	43.6	11.3	1.6
7.	Sodium Propionate (3-^{14}C)	1.14	15.8	37.6	28.4
8.	Sodium Propionate (2-^{14}C)	4.16	44.0	24.0	18.0
9.	Sodium Propionate (1-^{14}C)	33.30	42.3	10.6	8.1
10.	L-Methionine (S-$^{14}CH_3$)	0.5	16.9	24.7	22.0
11.	D,L-Tryptophan (7a-^{14}C)	2.7	20.8	2.4	<0.1
12.	D-Glucose (U-^{14}C)	0.54	16.5	6.1	1.3
13.	D-Glucosamine (1-^{14}C)	0.09	11.0	8.7	0.4
14.	Shikimate (U-^{14}C)	0.07	12.1	11.6	7.8
15.	D,L-Shikimate (1,6-^{14}C)	0.10	3.5	10.0	1.0
16.	Anthranilate (^{14}COOH)	0.02	24.0	3.0	<0.2
17.	Cinnamate (3-^{14}C)	0.08	8.6	3.2	<0.1
18.	Benzoate (^{14}COOH)	0.17	20.9	2.4	<0.1
19.	Salicylate (^{14}COOH)	0.25	15.0	4.9	<0.1

from C-2 and C-1 of the acetate, respectively. The results (Table 2) demonstrate that the second and third carbons of propionate are the source of the Me and adjacent methylenes of the spiroketal ring of A23187. The specific activity of the sodium acetate derived from propionate [3-^{14}C] and [2-^{14}C] labeled A23187 is one quarter that of the labeled antibiotic. This indicates that all four of the Me groups and adjacent methylenes of the diox-aspiro(5,5)undecane ring are derived from C-3 and C-2 of propionate, respectively. The results of the Schmidt degradation of the [3-^{14}C] propionate labeled acetates are also consistent with this proposal. The finding that 100% of the label that [3-^{14}C] and [2-^{14}C] propionate contribute to the antibiotic molecule is found at these carbons indicates that propionate is a direct precursor and not metabolically transformed prior to incorporation. The sodium acetate derived from the K-R oxidation of [2-^{14}C] and [1-^{14}C] acetate labeled A23187 methylester was also found to be labeled but the results are in accord with the metabolic conversion of acetate to propionate prior to its incorporation. The lack of incorporation of methionine, proline and shikimate and the low incorporation of glucose into this part of the molecule suggests that they are incorporated specifically into some other part of the molecule.

Direct evidence for the precursor labeling pattern of this antibiotic was obtained from feeding experiments with ^{13}C-labeled precursors in conjunction with ^{13}C-NMR. In order to do this, it was necessary to assign the ^{13}C-NMR spectrum of A23187. The free acid of the antibiotic was used for these studies. The ^{13}C-NMR spectrum of A23187 includes 29 clearly resolved resonances (Table 3-5). Of these, those due to the ketone and carboxyl groups have already received assignment.[6] To extend and complete these assignments, we have used a combination of routine methods. For example, the spectra of A23187 include 13 resonances below 95 ppm; from basic knowledge of ^{13}C chemical shifts, these resonances are assigned to the 12 sp^3 carbons plus the ketal (carbon 19). From the relaxation times (T_1) of these resonances and their appearances in the off-resonance decoupling (ORD) spectra, the five methine resonances are easily identified. These resonances were specifically assigned through the use of selective decoupling procedures.

The singlet resonances (i.e. those due to non-protonated carbons) of the aromatic region are more difficult to assign. The assignment of C(21) can be made through comparisons to the spectrum of 2-acetylpyrrole.[17] Appropriate models for the substituted benzoxazole are not available, but from the published spectrum of benzoxazole[18] itself and the use of substituent parameters drawn from extensive regressional analyses of benzenoid systems,[19] it is possi-

Table 2 Degradation of Specifically labeled A23187 Methyl esters

Precursor	Spec. Act. ($\times 10^2$) of A23187 ME (μCi/μmole)	Spec. Act. of Sodium Acetate from KRO $\times 10^2$ (μCi/μmole)	Schmidt Degradation % of Recovered Radio-activity in CH_3NH_2	CO_2	% of Total Precursor Label Found in Sodium Acetate
Proline (U-^{14}C)	4.14	0.003	N.D.	N.D.	0
Propionate-3-^{14}C	3.0	0.78	95.3	4.7	100
Propionate-1-^{14}C	2.4	0.54	5.8	94.2	100
Propionate-1-^{14}C	1.6	0	N.D.	N.D.	0
Acetate-2-^{14}C	1.9	0.064	56	44	30
Acetate-1-^{14}C	1.2	0.060	22	88	10
D,L-Methionine (S-$^{14}CH_3$)	1.1	0	N.D.	N.D.	0
D-Glucose (U-^{14}C)	0.61	0.01	N.D.	N.D.	10
Shikimate (U-^{14}C)	3.1	0.0004	N.D.	N.D.	0.29
D,L-Shikimate (1,6-^{14}C)	0.4	0.0002	N.D.	N.D.	0.3

N.D. - not determined

Table 3 Incorporation of [^{13}C] propionate into the Dioxaspiro(5.5)undecane ring of A23187 [a]

Carbon No.	(p.p.m.)	Relative ^{13}C Enrichment by Propionate [3-^{13}C][b]	[2-^{13}C][b]	[1-^{13}C][b]
8	166.1	0.7	0.7	1.1
9	32.3	0.9	0.8	1.0
10	68.3	0.8	0.5	14.9
11	28.7[c]	0.5	10.3	0.9
12	25.7	0.5	0.8	0.9
13	25.4	0.4	1.0	0.9
14	98.5	0.8	0.4	8.4
15	32.4	1.0	10.6	0.8
16	35.2	1.1	0.7	18.0
17	28.3[c]	0.5	10.4	0.8
18	22.8	0.7	0.5	14.2
19	42.5	0.6	11.6	0.8
11'	11.4[d]	10.8	0.7	1.0
15'	16.2	11.7	0.7	1.0
17'	10.8[d]	11.2	0.7	1.1
19'	13.2	12.1	0.6	1.0

[a]Calculated by dividing the peak intensity of C-3' into the intensities of the other carbon signals in the spectra of the enriched and natural abundance antibiotic. This relative signal height for the natural abundance carbon signals was divided into the corresponding carbon signals in the spectrum of the enriched antibiotic.

[b]The final concentration of C-13 labeled substrate added was 0.6 mg/ml. Spectra were recorded on ~ 20 mg of compound in 0.7 ml $CDCl_3$. Internal reference was tetramethyl silane.

[c,d]Resonances labeled with the same superscript may be interchanged in assignment.

Table 4

Carbon	PPM from TMS	Relative Enrichment[b] D,L-Proline-1-^{13}C (0.64 mg/ml)[c]	Relative Enrichment[b] L-methionine-S-$^{13}CH_3$ (0.8 mg/ml)
3'	30.0	1.0	16.0
11'	11.4[a]	1.0	1.4
15'	16.2	1.0	1.2
17'	10.8[a]	0.9	1.0
19'	13.2	0.8	0.9
20	193.2	7.0	0.7

[a] assignment may be interchanged.
[b] calculated as in table III except C-14 was used as the reference peak.
[c] final concentration of substrate added to production cultures.

ble to predict a spectrum for this moiety. Such a prediction, which was in reasonable agreement (< 2 ppm) with the previously assigned resonances of C-4 and -5, was used to provide an initial assignment of the singlet resonances of the benzoxazole portion of A23187.

Also useful in assigning the aromatic C resonances were the "long-range" (i.e. two- and 3-bond) ^1H-^{13}C couplings observed in the ^1H-coupled ^{13}C-NMR spectra. The resonances of C-8 and -1 could easily be distinguished using these data. H(4) is the nearest non-exchangeable proton to the carboxyl C-1, and inasmuch as these two nuclei are four bonds removed from each other, they are not expected to be significantly coupled. The C resonance near 165 ppm, which remains a sharp singlet in the ^1H-coupled ^{13}C-NMR spectrum, is therefore assigned to C(1). In the free acid, the resonance of C-8 is a triplet by virtue of its coupling to the adjacent methylene. This triplet is not resolved in the spectra of the complexes, presumably due to greater line widths resulting from the higher molecular weight of the ternary complexes (*Vide infra*), but peak widths can still be used to distinguish the resonances of these two carbons.

Two- and three-bond ^1H-^{13}C coupling can also be used to support the assignment of C (3, 6 and 7). The C(3) resonance is highly coupled, even after D exchange of the amine proton. Specific decoupling experiments using low decoupler power[20] confirmed that this resonance was coupled to the N-Me protons. The C(7) resonance appears as a doublet with line spacing of about 5.5 Hz, presumably resulting from coupling to H(5). The resonance of C(6) is apparently coupled to both H(4) and H(5), line spacings are about 1.2 and 3 Hz, respectively. Overlap of the C(2) resonance with those of other carbons prevents analysis of its appearance in the ^1H-coupled ^{13}C-NMR spectrum.

When a chloroform solution of A23187 is shaken with water or D_2O, changes in chemical shift such as seen in the spectra of narasin were not observed.[21] The ^1H-coupled ^{13}C-NMR spectra, however, showed numerous changes due to exchange of the two N-protons. All of the pyrrole carbons were observed to be coupled to the pyrrole N-H, a fact that aids in identifying the resonances of these carbons. Also, the C-4 resonance, which appears as a doublet of doublets in the spectrum of A23187, collapses to a

Table 5

Carbon	Chemical Shift, ppm	Relative Enrichment[a] Glucose [1-^{13}C] (2 mg/ml)	Relative Enrichment[a] Glucose [6-^{13}C] (2 mg/ml)
1	168.2	0.8	0.9
2	97.9	0.5	0.7
3	150.8	1.5	2.0
4	108.4	0.6	0.8
5	116.9	0.6	0.6
6	140.8	0.9	1.0
7	141.6	1.8	1.7

[a] calculated as in Table III.

doublet after deuterium exchange. Throughout the series, the resonance of C(5) is a sharp doublet and is not affected by D exchange.

Of the carbons of the aliphatic portion of the molecule, three are attached to O, one being a ketal which must be represented by the remaining downfield singlet at 98.5 ppm. The two CO carbons lead to doublet resonances at 68.3 and 72.8 ppm, assigned to C(10 and 18), respectively, through selective decoupling experiments.

The remaining upfield resonances can be subdivided into doublets, triplets, and Me resonances on the basis of T_1 and ORD experiments. Of the four doublet resonances, the lowest field must be due to C(19), which is adjacent to the ketone. This assignment was confirmed by selective decoupling. On the basis of the usual chemical shift correlations with β-substituent effects, the lowest field of the remaining doublets (32.4 ppm) is assigned to C-15. The remaining two doublets are not distinguished in assignment.

A23187 includes four methylene groups. The resonance of one of these (32.3 ppm) can be assigned through selective decoupling to C(9). Of the three remaining triplet resonances, the lowest field is assigned to C(16) through comparisons to model cyclohexane derivatives. The last two triplet resonances cannot be specifically assigned.

The N-Me carbon can be related to the resonance at 30.0 ppm by selective decoupling experiments. The upfield Me resonances (10.8 and 11.4 ppm) are assigned to the axial Me carbons at positions 11' and 17'.[22] The Me resonance at 16.2 ppm is assigned to the equatorial Me at C-15'. The fourth C-Me resonance, is assigned to the Me at position 19'.

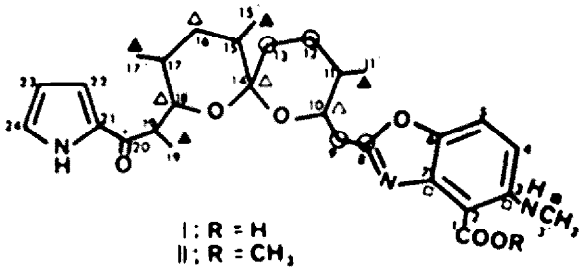
Once having assigned the ^{13}C-NMR spectrum of the antibiotic, conditions for feeding the ^{13}C-labeled precursors was first established by dilution experiments with the corresponding ^{14}C-labeled precursor. The stable isotope labeled compounds were then added to production cultures in divided doses at 72, 96 and 120 hr of cell growth at the concentrations indicated in Table 3.5. The antibiotic was isolated as before and the purified methyl ester was hydrolyzed with base to generate A23187 free acid.[23] The ^{13}C-NMR spectra of the enriched antibiotics was then examined.

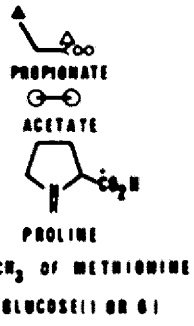
The incorporation of ^{13}C-labeled propionate revealed no surprises (Table 3). Propionate-3-^{13}C labeled only the Me carbons -11', -15', -17' and -19', of the diox-aspiro (5,5) undecane ring. Likewise, propionate-2-^{13}C labeled only the adjacent methylenes (11, 15, 17, 19) of this unit of the antibiotic and propionate-1-^{13}C enriched the C signals assigned to C (10, 14, 16 and 18). This leaves only four carbons unaccounted for in the diox-aspiro (5,5) undecane ring. As expected from the chemical degradation studies, acetate [2-^{13}C] and [1-^{13}C] were extensively metabolized by the organism prior to incorporation (data not shown). In order to determine where intact acetate units were incorporated, acetate [1, 2-$^{13}C_2$] was fed to the antibiotic producing *S. chartreusis*. This enriched A23187 revealed two pairs of carbons derived from intact acetate units as determined by the presence of only two sets of coupled carbons. C-8 and -9 were coupled to each other ($^1J_\alpha$ = 59.6 Hz) and C-12 and -13 ($^1J_\alpha$ = 24 Hz) were also coupled. Therefore, acetate was being incorporated specifically into these two sets of carbons only. This confirms the earlier suggestion[11] that the spiroketal ring is formed by the condensation of four propionate and two acetate units.

The role of proline and methionine in the biosynthesis was established in a similar fashion (Table 4). Proline [1-^{13}C] was specifically incorporated into the C-20 of A23187 and likewise, L-methionine (Me ^{13}C) was incorporated only into the amino Me group of the C_1N_1 unit. No other C resonances demonstrated enrichment in the spectra of either enriched antibiotic.

The origin of the C_1N_1 unit of A23187 still remained to be determined. The lack of incorporation of anthranilic acid (^{14}COOH), tryptophan [7a-^{14}C] and the other aromatic (Table 1) containing compounds suggested that this aromatic unit might be derived more directly from glucose metabolism. The finding that intact acetate units labeled only the carbons of spiroketal ring also ruled out the possibility that acetate units condensed to form this moiety. Another possibility for the origin of this unit from glucose would be via a shikimate-type pathway. Feeding experiments with D-glucose [1-^{13}C] and [6-^{13}C] revealed that only two carbons of the C_1N_1 unit became labeled from these substrates (Table 5). The carbons enriched were *ortho* to the exocyclic C in agreement with the involvement of a shikimate-type pathway in the biosynthesis.[24]

Although the results with D-glucose [1-^{13}C] and [6-^{13}C] suggest the involvement of a shikimate-type of pathway, the data cannot distinguish between three possible pathways for the formation of the C_1N_1 unit (Scheme 2).[25] D-glucose [U-$^{13}C_6$] was used to determine how the intact three C unit from phosphoenolpyruvate and one intact four C unit from erythrose-4-phosphate condense to form the C_1N_1





Scheme 1

Table 6. ^{13}C-^{13}C coupling of carbons of the C_7N_1 unit of A23187 methylester labeled from [U-$^{13}C_6$] glucose

Carbon	Chemical Shift, ppm	J_{CC}, Hz
1	168	76
2	100.5	76, 74
3	150.8	62
4	108.4	62,63
5	116.9	63,66
6	142.5	66
7	142.3	74

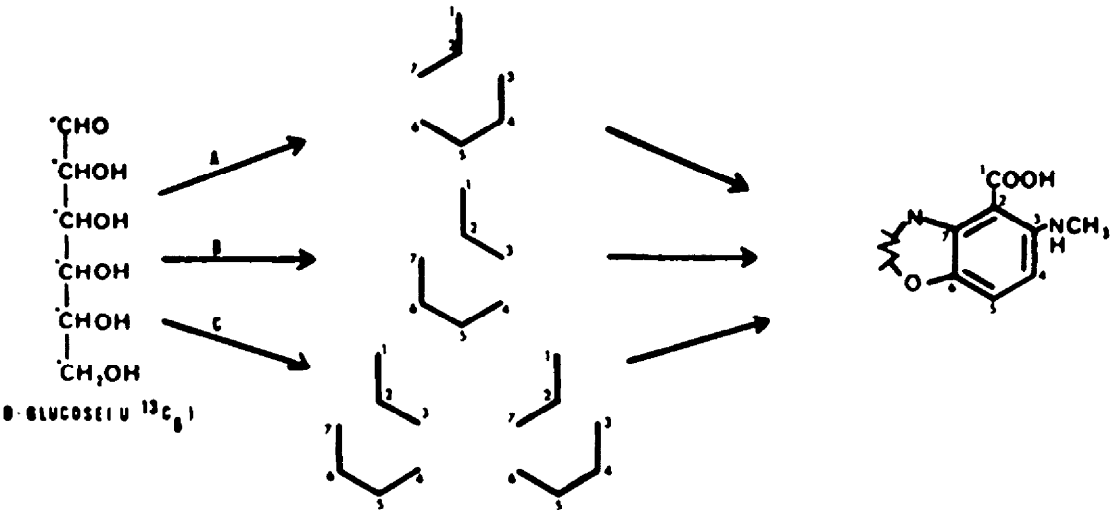
unit. Pathways A and B (Scheme 2) differ in the orientation of the two units. Pathway C would occur if a free symmetrical intermediate such as 2,6-diaminobenzoic acid were involved in the biosynthesis. This pathway could give rise to a more complicated coupling pattern since C-2 would be coupled to C-7 and C-3. D-glucose [U-$^{13}C_6$] was diluted with unlabeled glucose (0.25 gm labeled to 0.75 gm unlabeled) and fed to 10 production cultures of *S. chartreusis*. The couplings found in the C-13 NMR spectrum (Table 6) of the enriched methylester of A23187 (II) are straight forward and consistent with the involvement of pathway A in the biosynthesis. The resonance for C-2 is spanned by a multiplet (J_α = 74 and 76 Hz) and is coupled to C-1 (doublet, J_α = 76 Hz) and C-7 (J_α = 74 Hz). The resonance at C-3 is spanned by a doublet (J_α = 62 Hz) and is coupled to C-4 (multiplet, J_α = 62 and 63 Hz) which is also coupled to C-5 (multiplet, J_α = 63 and 66 Hz). Finally, C-5 is coupled to C-6 (doublet, J_α = 66 Hz). These results are only in accord with the involvement of Pathway A in the biosynthesis of this unit.

Shikimic acid [^{14}C] is significantly incorporated into A23187 (Table 1). This precursor is apparently specifically incorporated into the C_7N_1 unit since K-R oxidation of shikimate-[U-^{14}C] labeled A23187 indicated that less than 0.6% of the label that shikimate contributes to the antibiotic ends up in the diox-aspiro (5,5) undecane ring (Table 2).[23] The addition of unlabeled proline along with shikimate [U-^{14}C] did not decrease the incorporation of shikimate into the antibiotic suggesting that none of the label that shikimate was contributing to the antibiotic was due to metabolism and subsequent incorporation into the α-ketopyrrole unit. The results with specifically C-13 labeled glucose and shikimate [^{14}C] taken together indicate that the C_7N_1 unit of A23187 is formed by a divergence of the shikimate pathway at a point beyond the formation of shikimic acid itself.

DISCUSSION

The results presented above establish the biosynthetic origin of antibiotic A23187 and are summarized in I. The precursors for this antibiotic are proline, propionate, acetate and glucose via a shikimate-type pathway. Proline forms the α-ketopyrrole moiety while acetate and propionate are utilized to construct the diox-aspiro (5,5) undecane ring. This parallels the biosynthesis of macrolide[24] and ansamycin antibiotics where Me groups extending from the chain arise from methyl malonate (propionate), instead of requiring a one C unit donated from methionine as is found in aurodox[10] and boromycin.[11] As with the ansamycins, the chain of the diox-aspiro (5,5) undecane ring is elongated in an "amide-head" direction in which the chain is initiated starting with the N of the aromatic unit. In the case of A23187, this would be an "oxazole-head" pathway. Instead of ending with the condensation of an acetate unit or propionate unit, the chain extension would terminate with the addition of proline or pyrrole-1-carboxylate. Since the addition of unlabeled pyrrole-2-carboxylate along with D,L-proline[1-^{14}C] did not reduce the incorporation of proline into the antibiotic (data not shown), it is likely that the last methylmalonate (propionate) unit condenses directly with proline which then is reduced to the α-ketopyrrole moiety.

The C_7N_1 unit differs from the C_7N_1 units found in a number of interesting and clinically useful antibiotics[14] in that shikimic acid serves as a precursor for this portion of the antibiotic. The divergence of the pathway for this unit must occur prior to the formation of anthranilic acid or 3-hydroxy anthranilic acid since anthranilic [COOH] acid and tryptophan (7a-^{14}C), respectively, were not incorporated into the antibiotic. Phenylalanine [U-^{14}C] and tyrosine [U-^{14}C] were found to label the antibiotic (about 1% of the added label), but chemical degradation of the specifically labeled A23187 revealed that 80% of this label was being randomly incorporated into the spiroketal ring (data not shown). These results suggest that the nitrogen functionalities of the ring are added to this unit between shikimic acid and the aromatization of the ring. The C_7N_1 functionalities of the ansamycins, mitomycins[14] and pactamycin, are thought to have 3-dehydroquinate (DHQ) or 3-dehydroshikimate (DHS) as the immediate precursor for this portion of the molecule (Scheme 3). This is indicated by the lack of specific incorporation of shikimate (III) into these units.[25,10] An attractive possibility for the introduction of an amino group into these C_7N_1 unit would be transamination of the keto group of DHS or DHQ. Since the nitrogens of the C_7N_1 units of A23187 are *ortho* to the exocyclic C, a similar type of mechanism would seem unlikely in this particular biosynthesis. The generation of the C_7N_1 unit of A23187 may require that shikimate be converted to 5-enolpyruvyl-shikimate-3-phosphate (IV) or some other



Scheme 2

intermediate and then to 2,6-diaminobenzoic acid or 3-hydroxy-2,6-diaminobenzoic acid (VII) via a divergence of the pathway to anthranilate (X). A similar but separate pathway may be involved in generating the C_6N_2 unit of streptonigrin (IX).[30] Recently, 3-amino-5-hydroxybenzoic acid (V) has been demonstrated to be a true intermediate in the formation of the ansamycins[27] and mitomycins,[28] and 3-aminobenzoic acid (VI) was found to efficiently label pactomycin.[24] Our results with D-glucose [U $^{13}C_6$] and the finding that the addition of unlabeled 2,6-diaminobenzoate along with shikimic acid [U ^{14}C] did not specifically reduce the incorporation of this labeled precursor[15] would suggest that 2,6-diaminobenzoic acid is not a free intermediate in the biosynthesis of the C_6N_2 unit. Currently, studies are underway to determine more directly the role of a shikimate pathway in the biosynthesis of this unit and the intermediacy of 3-hydroxy-2,6-diaminobenzoic acid (VII).

EXPERIMENTAL

General methods. ^{13}C-NMR spectra were recorded on a JEOL FX-270 multinuclear magnetic resonance spectrometer operating at 67.8 MHz. Assignment of spectra was carried out at The Lilly Research Laboratories. ^1H-NMR spectra were recorded on a Varian HA-100 spectrometer while ^{13}C-NMR spectra were recorded on a JEOL PFT-100 spectrometer interfaced with an EC-100 data system. Chemical shifts are given relative to internal Me_4Si.

Chromatography for purification of A23187 was carried out on preparative TLC plates (Merck, 60F-254, 0.5 mm thickness) or on columns of silica gel (Baker, 80-200 mesh). Radioisotope labeled compounds were purchased from New England Nuclear, Amersham-Searle, Research Products and ICN. Radioactivity was determined by liquid scintillation counting on a Beckman LS-9000 or LS-7800 LSC spectrometer using 10 ml of aquasol (NEN). Counting efficiencies were determined by the external standardization. ^{13}C-labeled precursors were purchased from Merck and all precursors were at least 90% enriched with ^{13}C. Kuhn-Roth oxidation and Schmidt degradation were carried out as described elsewhere.[16]

Culture conditions. Streptomyces chartreusis (NRRL 3882) was maintained on sterile soil cultures. A loopful of this sterile soil culture was inoculated into flasks (250 ml. Erlenmeyer) containing 50 ml of a seed medium consisting of 1.5% dextrose, 1.5% nutrisoy flour (Staley), 0.5% corn syrup solids (Staley), 0.2% $CaCO_3$ and 0.5% NaCl adjusted to pH 7.0. The seed culture was allowed to grow for 2 days on a rotary shaker (250 rpm) at 25°. From this seed culture, 2 ml samples were transferred to 250 ml Erlenmeyer shake flasks containing 50 ml of the production medium. The production medium consisted of 0.4% soybean meal (Brookfield), 0.1% casein, 2% dextrose and 0.3% sodium nitrate adjusted to a final pH of 7.3.

Isolation of A23187. After the appropriate growth time in production medium, cultures were harvested by filtering off the mycelium. The mycelia were washed with water and then extracted with hot MeOH. The broth also possessed some of the antibiotic and it was adjusted to pH 10.0 and extracted with EtOAc. The extracts were combined and evaporated to near dryness. The residue was dissolved in 0.1 N HCl and shaken with an equal volume of EtOAc (twice). The EtOAc extracts were combined and extracted with an additional amount of 0.1 N HCl. This was found to effectively convert all of the A23187 Ca-Mg mixed salt to the free acid of the antibiotic. The organic layer was then dried with Na_2SO_4 and evaporated. The crude dried extract was treated with diazomethane in ether. The A23187 methyl ether (II) was purified either on preparative TLC plates in benzene–EtOAc (50:50) or by column chromatography in benzene–EtOAc (90:10). Hydrolysis of the A23187 methyl ester to the free acid was carried out with ethanolic KOH. The reaction was started by adding 3 ml of 10% KOH soln to 50 ml of EtOH containing 50 mg of A23187 methyl ester. The mixture was heated at 60° for 3 hr and then cooled to room temp. Water (120 ml) was added and the EtOH removed under vacuum. The pH was adjusted to 7.0 and the hydrolysis product extracted with EtOAc. The Ca salt was prepared by shaking the organic layer over 1.0 m Tris containing 0.5 m $CaCl_2$. The organic layer was dried with $NaSO_4$ and then evaporated (A23187). Ca^{++} was purified by column chromatography and the purified antibiotic converted to its free acid. This procedure was necessary to remove contaminating material which was not removed by direct purification of A23187 free acid or its mixed salts.

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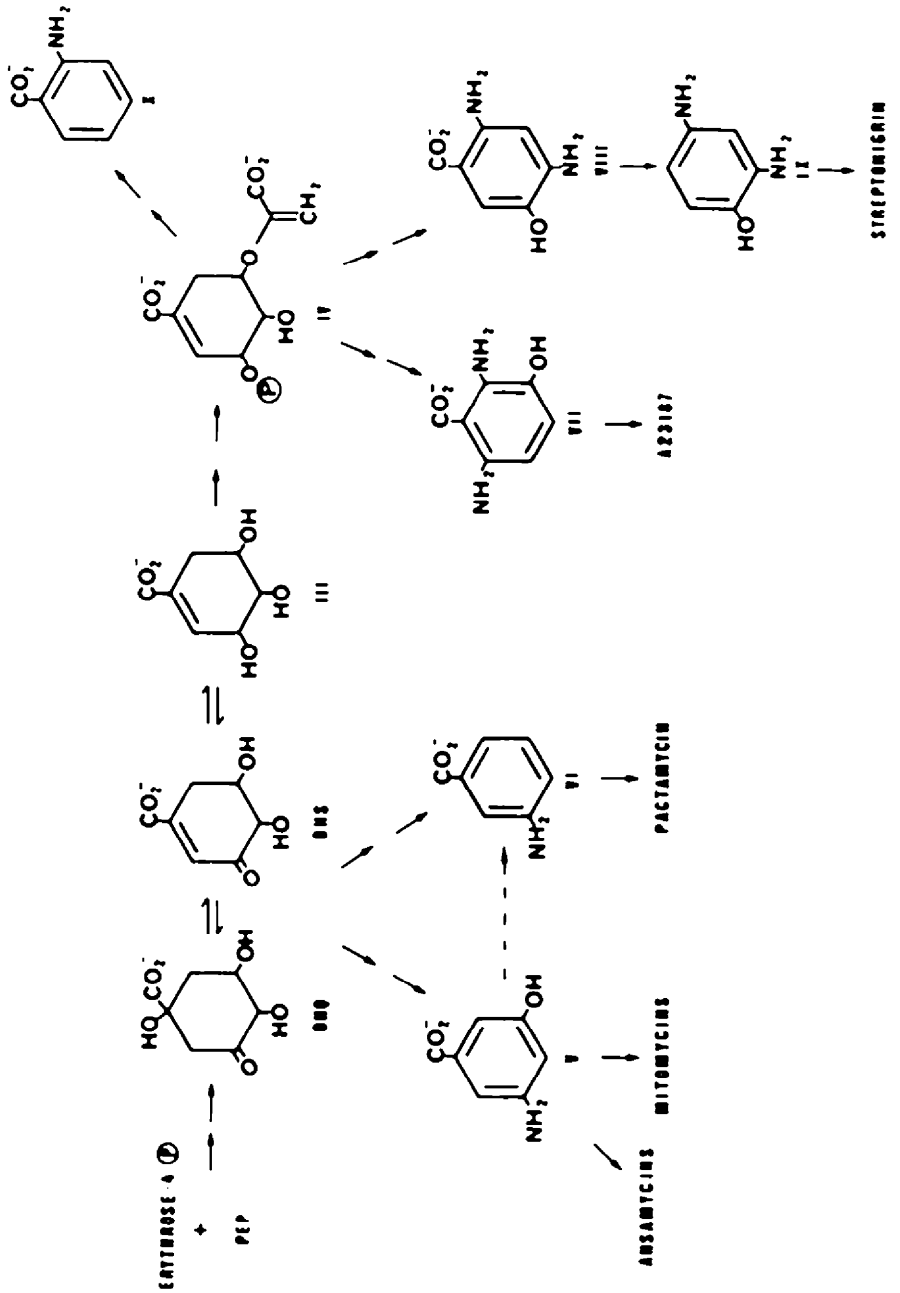

Scheme 3 Postulated pathways to C_6N_1, C_6N_2, and C_6N_3 moieties

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