AROMATIC BIOSYNTHESIS

III. Role of *p*-Aminobenzoic Acid in the Formation of Vitamin B_{12}[1]

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A preceding paper in this series (Davis, 1951) described mutants of *Escherichia coli* that require 5 growth factors: tyrosine, phenylalanine, tryptophane, *p*-aminobenzoic acid (PABA), and *p*-hydroxybenzoic acid (POB). These quintuple aromatic auxotrophs are blocked at various stages in aromatic synthesis; those with the earliest block (e.g., strain 83-1) are satisfied in their growth requirement by a single nonaromatic intermediate, shikimic acid (a 3,4,5-trihydroxycyclohexenecarboxylic acid), or by its precursor, Compound X.

In applying these mutants to the problem of aromatic biosynthesis it seemed likely that all aromatic metabolites might arise through a common path. A mutant blocked early enough in aromatic synthesis would be unable to make any of these compounds unless supplied with a precursor of it, or a compound that the cell can convert to a normal precursor. It therefore appeared significant that the quintuple auxotrophs do not require, and are not even accelerated by, riboflavin, menadione (2-methyl-1,4-naphthoquinone), or vitamin B_{12} (which yields 5,6-dimethylbenzimidazole on degradation (Brink and Folkers, 1949)). Of these compounds, B_{12} is a known metabolite of *E. coli* (Davis and Mingioli, 1950); its aromatic portion therefore must be derived either from one of the components of the quintuple supplement or else outside the X-shikimic path. The position of riboflavin and menadione is less clear, since these compounds have not been shown to affect *E. coli* when supplied externally; despite extensive search, no mutants requiring either compound have been obtained. Furthermore, these substances, unlike B_{12} (see later), do not spare any component of the quintuple supplement.

This paper will present evidence, based on sparing action, that one of the components of the quintuple supplement, PABA, participates in the synthesis of B_{12}. It will further point out that the relative requirement of *E. coli* for these two vitamins is equally compatible with the function of PABA as a catalyst or as a structural precursor in B_{12} synthesis. Therefore, the absence of a B_{12} requirement in the quintuple aromatic auxotrophs is due conceivably to derivation of the aromatic portion of B_{12} from PABA.

Two other aspects of PABA metabolism also will be considered: the mediation of B_{12} in the known relation of PABA to methionine synthesis, and the reported role of folic acid in thymine synthesis in *E. coli*.

[1] Paper II in this series (Davis, 1950) was inadvertently not listed as such in publication.

EXPERIMENTAL METHODS

The isolation of the mutants and the cultural methods have been described in previous publications (Davis, 1949, 1951). Medium A (Davis and Mingioli, 1950), which was used, contains glucose (autoclaved separately) and citrate as sole organic constituents.

For quantitative measurement of growth response of a PABA auxotroph, a 48 hour culture was washed with medium A and diluted 1:100 in 10 per cent glucose; 0.2 ml was inoculated into 10 ml of medium A (without glucose) that had been supplemented as indicated and autoclaved in colorimeter tubes seven-eighths of an inch in diameter. After incubation at 37 C light transmission was measured in an Evelyn photoelectric colorimeter, using a 620 mμ filter. Experiments with the quintuple auxotrophs were performed similarly except that the medium was supplemented with an excess of the known requirements other than PABA; in addition, the assay with these strains was improved by the use of 0.5

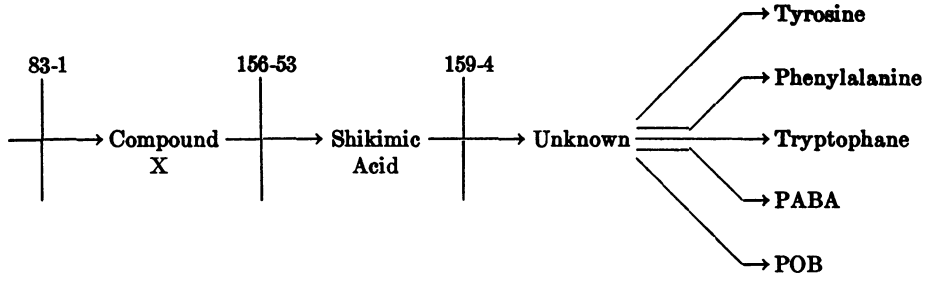

Figure 1. Sites of blocks in quintuple aromatic auxotrophs.

rather than the usual 0.2 per cent glucose. Tests at the end of several experiments showed that the turbidity was due entirely to the mutants and not to prototrophic reversions.

RESULTS

Absence of B_{12} requirement in quintuple auxotrophs. We have noted elsewhere that strain 83-1 and a number of similar mutants grow slowly on a quadruple aromatic supplement of tyrosine, phenylalanine, tryptophane, and PABA (Davis, 1950, 1951); these strains clearly do not have an absolute requirement for vitamin B_{12}, despite its aromatic structure. Since POB could be readily shown to accelerate the growth of these strains (Davis, 1950), B_{12} was tested similarly on solid media for the possibility of a relative requirement; no acceleration was observed in the presence of either the quadruple or the quintuple supplement.

It seemed unlikely that this absence of demonstrable acceleration could be due to the traces of B_{12} in the agar, for B_{12} auxotrophs grow very little on these media (Davis and Mingioli, 1950). In order to exclude this possibility and to test more sensitively for acceleration, growth was measured quantitatively in tubes with the following quintuple auxotrophs (figure 1): strain 83-1, which responds to Compound X or shikimic acid; strain 156-53, which accumulates

Compound X and responds to shikimic acid; and strain 159-4, which accumulates shikimic acid.

Table 1 shows that B_{12} causes no significant acceleration in the presence of either the quadruple or the quintuple supplement, confirming the results previously obtained on solid media. The accelerating effect of POB is included in table 1 in order to demonstrate the sensitivity of the method.

Sparing of PABA requirement by vitamin B_{12}. The failure of the quintuple auxotrophs to require B_{12} suggested that the aromatic portion of this vitamin might be derived from one of the compounds already present in the quintuple supplement. It therefore seemed possible that B_{12} might spare part of a require-

TABLE 1

Acceleration of growth of quintuple auxotrophs by POB, but not by vitamin B_{12}

Medium: "A" (see "Methods") with 0.5 per cent glucose, supplemented with L-tyrosine 20 μg per ml, DL-phenylalanine 40 μg per ml, and L-tryptophane 10 μg per ml.

Inocula: 5×10^{-3} ml of cultures grown 48 hours on same medium plus 1 mμg per ml PABA.

mμg/ml				Per cent light transmission					
				Strain 83-1		Strain 156-53		Strain 159-4	
PABA	POB	B_{12}	Shikimic Acid	18 hours	24 hours	18 hours	24 hours	18 hours	24 hours
—	—	—	—	99	99	99	99	99	99
10	—	—	—	87	69	94	91.5	89	73
100	—	—	—	—	—	95	92	90	76
10	10	—	—	67	52	76	45	62	50
10	—	10	—	87	69	91	82	88	74
10	10	10	—	61	52	81	48	65	48
10	10	—	100*	53	54	46	45	65	48

* The accelerating effect of shikimic acid much exceeds that shown in the table; full growth of strains 83-1 and 156-53 was reached in its presence well before 18 hours. Strain 159-4 is blocked after shikimic acid (figure 1), and hence is not accelerated by it.

ment of strain 83-1. Such an effect on the PABA requirement was readily demonstrated.

This effect was demonstrated initially on solid media; early efforts to extend the study quantitatively with tubes were unsuccessful because inocula of this mutant, grown in the usual manner in the presence of an excess of PABA, yielded appreciable turbidity on cultivation without PABA, and quite heavy growth with the addition of B_{12}. Similar results were obtained with PABA auxotroph 48A-33. This capacity for growth without added PABA probably is due to intracellular storage of PABA or its derivatives: though the effect could not be eliminated by washing the cells, it was prevented by cultivating the inoculum for 48 hours in the presence of a growth-limiting amount of PABA (1 mμg per

ml). Accordingly, subsequent experiments with both single and quintuple auxotrophs were conducted with such inocula, presumably free of stored PABA.

The results of one of several experiments in liquid media, presented in table 2, show that vitamin B_{12} produces a marked increase in growth of strain 83-1 in the presence of limiting amounts of PABA. The similar effect of methionine will be discussed later.

The interpretation of these data is complicated by the fact that the quintuple auxotrophs have additional unknown relative requirements, as shown by their acceleration on addition of shikimic acid (table 1). Furthermore, a mutant with

TABLE 2

Sparing effect of B_{12} or methionine on PABA requirement of quintuple auxotroph
Medium: as in table 1, with further supplement of POB 0.01 μg per ml.
Inoculum: strain 83-1, as in table 1.

SUPPLEMENT			PER CENT LIGHT TRANSMISSION	
PABA mμg/ml	B_{12} mμg/ml	DL-Methionin μg/ml	24 hours	48 hours
0	—	—	99	99
0.1	—	—	98	97
0.3	—	—	92	89
1	—	—	74	66
3	—	—	56	55
10	—	—	54.5	53.5
0	10	—	99	97.5
0	—	20	97.5	96
0.1	10	—	91.5	81
0.1	—	20	92	62
0.3	10	—	82	68
0.3	—	20	75	60
10	10	—	52	53
10	—	20	53	52

an early block of this type could theoretically allow diversion of PABA into other aromatic metabolites by reversal of a normal biosynthetic process; this possibility should not exist in a mutant blocked specifically before PABA. For these reasons quantitative data on sparing action were also obtained with PABA auxotroph 48A-33. This strain has the further advantage of being exceptionally stable, allowing prolonged incubation without the emergence of reversions.

One of several similar turbidimetric experiments with the PABA auxotroph is presented in table 3; similar results were obtained on solid media. It is seen that with this mutant, as with the quintuple auxotrophs, B_{12} exerts a marked sparing and accelerating effect in the presence of a limiting amount of PABA. All the other available water-soluble vitamins, including riboflavin, menadione, pyridoxal, nicotinamide, and meso-inositol also were tested; the results were negative.

The sparing effect shown in tables 2 and 3 might be simply a reflection of the acceleration produced by B_{12}, since turbidity had not become constant by 48 hours. (The continued slow growth on limiting amounts of PABA will be discussed later.) One experiment with the stable PABA auxotroph was therefore continued for 5 days; by this time a plateau of turbidity had been reached, and the sparing effect of B_{12} persisted.

Sparing of PABA requirement by other compounds. Methionine is known to antagonize sulfonamide inhibition noncompetitively (Bliss and Long, 1941) and

TABLE 3

Sparing effect of B_{12} on requirement of PABA auxotroph

Medium: "A" with 0.2 per cent glucose.

Inoculum: 2×10^{-3} ml of strain 48A-33 grown 48 hours in same medium plus PABA 1 mμg per ml.

SUPPLEMENT	PABA: mμg/ml													
	0		0.1		0.2		0.5		1.0		2.0		10.0	
	Hours of Incubation													
	24	48	24	48	24	48	24	48	24	48	24	48	24	48
	Per Cent Light Transmission													
None	100	100	99.5	97	97	85	82	71	63	60.5	55	52.5	56.5	50
B_{12}, 0.5 mμg per ml	100	98	97	77	77	72.5	62.5	61	—	—	—	—	—	—
B_{12}, 10 mμg per ml	99.5	95	96.5	76	76.5	70.5	62	60	—	—	—	—	—	—
DL-Methionine, 2 μg per ml	100	95	98	85	85	80	74	70	—	—	—	—	—	—
DL-Methionine, 20 μg per ml	98	94.5	98	68	90.5	64	67	63	—	—	—	—	—	—
Mixture B*	100	100	97	84	90.5	79.5	70	66.5	—	—	—	—	—	—
Mixture B + B_{12}, 1 mμg per ml	98.5	75	83	69.5	69.5	66	57	57.5	—	—	—	—	—	—
Mixture B + methionine, 20 μg per ml	95	67.5	82	63	60	57	56	54	—	—	—	—	—	—

* Mixture B consisted of compounds other than methionine that are known to antagonize sulfonamides noncompetitively; these tubes contained L-serine 50 μg per ml, DL-valine 10 μg per ml, adenine 10 μg per ml, hypoxanthine 10 μg per ml, and thymine 10 μg per ml. L-serine was used rather than the DL compound because of the toxicity of D-serine for this strain of *E. coli* (Davis and Maas, 1949).

spare the requirement of a PABA auxotroph (Lampen *et al.*, 1949). Its PABA-sparing effect would be expected to resemble that of B_{12}, in view of the equivalence of these compounds as sulfonamide antagonists (Shive, 1950; Davis and Mingioli, 1950) and growth factors for B_{12} auxotrophs of *E. coli* (Davis and Mingioli, 1950). Confirming this expectation, table 2 shows with a quintuple aromatic auxotroph, and table 3 with a PABA auxotroph, that methionine in excess (20 μg per ml) spares PABA to an even slightly greater extent than does B_{12} in excess.

PABA sparing was also observed with a mixture of other known noncompetitive sulfonamide antagonists, confirming the results of Lampen *et al.* (1949).

These compounds include purines, thymine, serine, and valine (Winkler and de Haan, 1948; Winkler *et al.*, 1949). Table 3 shows that this mixture (B) spares PABA somewhat less than does either B_{12} or methionine. The combination of mixture B plus either B_{12} or methionine is much more effective, supporting quite heavy growth in the absence of added PABA, yet even under these circumstances growth is accelerated further by the addition of as little as 0.1 mμg per ml of PABA, a concentration too low to support appreciable visible growth alone. (For other PABA-sparing compounds cf. Winkler and de Haan, 1948; Lampen *et al.*, 1949.)

Wooley (1951) has suggested, on the basis of the antagonism of 1,2-dimethyl-4,5-diaminobenzene and *o*-phenylenediamine to the antibacterial action of 1,2-

TABLE 4

Failure of folic acid (PGA) to substitute for thymine in sparing PABA

Experimental conditions as in table 3. The failure of PGA to substitute for thymine was also observed when mixture C was further supplemented with methionine.

SUPPLEMENT	PABA: mμg/ml											
	0			0.1			0.2			0.5		
	Hours of Incubation											
	24	48	72	24	48	72	24	48	72	24	48	72
	Per Cent Light Transmission											
None	100	99.5	99	99	96	91	98	90.5	85.5	88.5	78	76
PGA 0.1 μg per ml	100	99.5	97.5	99.5	96.5	91	97.5	90.5	85.5	89	78	76.5
Mixture C*	100	100	98	100	95	93	95.5	92	90	82	76	77
Mixture C + thymine, 10 μg per ml	100	99	98	100	96	85	94.5	81.5	81	81.5	72	71
Mixture C + PGA, 0.1 μg per ml	100	100	98	100	96.5	93	95	92	90	81	76	76

* Mixture C: L-serine 50 μg per ml, DL-valine 10 μg per ml, hypoxanthine 10 μg per ml, uracil 10 μg per ml.

PGA: filter-sterilized solution of a highly purified aldehyde-free sample of pteroylglutamic acid, generously furnished by Dr. T. H. Jukes of Lederle Laboratories.

dichloro-4,5-diaminobenzene, that the first of these compounds, known to be a degradation product of B_{12}, may act as an intermediate in the biosynthesis of B_{12} as well as that of riboflavin. Accordingly, 1,2-dimethyl-4,5-diaminobenzene,[2] 5,6-dimethylbenzimidazole[2] (another degradation product of B_{12}), and *o*-phenylenediamine were tested (as in table 3) at concentrations of 10, 100, and 1,000 mμg per ml for possible sparing of PABA requirement. No effect was seen except that 5,6-dimethylbenzimidazole was inhibitory at the highest concentration.

Absence of response to pteroylglutamic acid (folic acid) or citrovorum factor. It is well known that pteroylglutamic acid (PGA), a PABA derivative, fails to antagonize sulfonamide inhibition in *E. coli* and many other bacterial species.

[2] Furnished through the courtesy of Dr. K. Folkers of Merck and Company.

Similarly, PGA even in huge concentrations does not support the growth of a PABA auxotroph of *E. coli* (Lampen *et al.*, 1949). In light of the proposed conversion of PABA to B_{12}, it seemed possible that the inability of PGA to replace PABA might be due to a limited function of PGA as only one of the PABA derivatives in the cell. Mixtures of PGA and B_{12}, however, tested over a wide concentration range, failed to support growth of the PABA auxotroph. Furthermore, PGA did not spare the PABA requirement. The inactivity of PGA therefore remains unexplained.

Woods (1950) has proposed a general theory of PABA function, part of which is based on the report that PGA can substitute for thymine but not for any other noncompetitive sulfonamide antagonists in *E. coli* (Winkler and de Haan, 1948). Shive (1950) has also remarked that PGA and thymine are somewhat interchangeable. In view of the parallel behavior of various other compounds in antagonizing sulfonamides and sparing a PABA requirement, one would expect PGA to spare PABA under the same circumstances that permit thymine to show this effect. With a highly purified sample of PGA, however, even in great excess, we could not observe the PABA-sparing action shown by thymine (table 4). It is therefore concluded that a relationship of PGA to thymine synthesis in *E. coli* has not been established. Since thymine and PGA were reported to be active against sulfonamides only in the presence of several other sparing compounds, including methionine, and since these conditions markedly sensitize the cells to traces of PABA, it seems possible that the positive results reported may have been caused by PABA present in the PGA or released during incubation (cf. Koft *et al.*, 1950). With the mutants it has been possible to show a PABA-sparing effect of thymine even in the absence of methionine (table 4).

Since citrovorum factor is closely related to PGA, it was tested for its ability to support the growth of a PABA auxotroph of *E. coli*. The activity of a concentrate of naturally occurring material[3] was so low (1 per cent that of PABA) that it was probably due to impurity.

DISCUSSION

It has been shown that vitamin B_{12} spares the PABA requirement of mutants of *E. coli*. This effect is taken to imply that PABA participates in B_{12} synthesis, as might also be inferred from the noncompetitive antagonism of sulfonamide inhibition by B_{12} (Shive, 1950; Davis and Mingioli, 1950). While the function of PABA in the synthesis of other PABA-sparing compounds is clearly catalytic, the data for B_{12} are equally compatible with a role of PABA as a structural precursor: the respective requirements of B_{12} and PABA auxotrophs of *E. coli* are 0.5 mμg per ml (Davis and Mingioli, 1950) and 2 mμg per ml (table 3), the resulting molar ratio of B_{12}/PABA being only 1/50.

Further indirect evidence on the nature of the relation of PABA to B_{12} is furnished by the observation that B_{12} is not required by the quintuple aromatic auxotrophs. This fact, however, also permits at least two explanations: the aromatic portion of B_{12} might arise from Compound X and shikimic acid via some compound already present in the quintuple supplement, such as PABA,

[3] Kindly furnished by Dr. T. H. Jukes of Lederle Laboratories.

or else these two key intermediates might not be precursors of B_{12} at all. (The latter possibility could involve synthesis of B_{12} either by an entirely independent path or by branching off the same path before the block in 83-1.)

The extent of PABA-sparing action cannot be measured precisely since PABA, as a catalytic growth factor, does not provide the sharp threshold of growth responses seen with assays for "building-blocks" such as amino acids or purines; on the contrary, growth continues slowly for several days after initial rapid growth in the presence of limiting amounts of PABA. While it is possible that these mutants are incompletely blocked and hence slowly synthesize PABA, it seems more likely, since a plateau of growth is eventually reached in prolonged experiments, that some growth can take place even when it causes dilution of the intracellular PABA or its derived coenzymes to suboptimal levels.

As a fair approximation it can be concluded from tables 2 and 3 that under some circumstances B_{12} or methionine triples the growth response to a small amount of PABA. This figure parallels the observation of Shive and Roberts (1946) that the inhibition index of methionine is three times that of the purines, which occupy the next position in the series of sulfonamide antagonists. If PABA acts as a structural precursor of B_{12}, one might wonder how the replacement of a stoichiometrically trivial fraction (1/50) of the PABA requirement of the cell could exert so much sparing action. This problem, however, is even more striking if PABA functions catalytically in B_{12} synthesis. Whichever the mechanism, the explanation, provided by studies on antagonism to sulfonamides (Shive and Roberts, 1946; Shive, 1950), appears to be that the intracellular level of PABA required for the synthesis of methionine (via B_{12}) is greater than that required for the other PABA-dependent syntheses. The extent of the sparing action therefore would bear no simple stoichiometric relation to the amount of the sparing compound required.

The PABA-sparing action of B_{12}, its failure to be required by quintuple aromatic auxotrophs, and the quantitative requirements of the organism for the two compounds are all consistent with the structural conversion of a small fraction of the PABA in the cell to B_{12}. This interpretation seems especially plausible since considerations of economy would favor a single path of aromatization in the cell, and the nonaromatic structure of shikimic acid and Compound X[4] assigns to the quintuple auxotrophs a very early block in the known path. Nevertheless, none of the available evidence can exclude catalytic action of PABA in the synthesis of B_{12} from a precursor other than Compound X. Accordingly, more direct testing of the possible conversion of PABA to B_{12} is planned.

It has been shown that B_{12} and methionine exert a similar maximal sparing effect on PABA requirement; this result is in harmony with their previously demonstrated equivalence as noncompetitive antagonists of sulfonamide inhibition (Shive, 1950; Davis and Mingioli, 1950), and as growth factors for a mutant of *E. coli* (Davis and Mingioli, 1950). Furthermore, with either compound all three effects become maximal at the same concentration. Since B_{12} is concerned with the synthesis of methionine (Davis and Mingioli, 1950), and

[4] Isolated and identified as a dehydroshikimic acid (Salamon and Davis, 1951).

PABA with the synthesis of B_{12}, the antisulfonamide and PABA-sparing action of methionine appear to depend on its replacement of a B_{12} requirement.

Elsewhere we have presented evidence that the site of action of B_{12} in methionine synthesis is the methylation of homocysteine (Davis and Mingioli, 1950). PABA, as a participant in B_{12} synthesis, must be indirectly involved in the same reaction. This work therefore confirms the conclusion, derived from studies on sulfonamide antagonism (Harris and Kohn, 1941; Winkler and de Haan, 1948; Shive, 1950) and on a PABA auxotroph of Neurospora (Zalokar, 1950; Strehler, 1950), that PABA is involved in the methylation rather than the formation of homocysteine; our data do not support the opposite conclusion of Lampen *et al.* (1949) regarding PABA, or Dubnoff (1950) regarding B_{12}.

The slightly greater PABA-sparing effect of methionine compared with B_{12} (tables 2 and 3) suggests that B_{12} may not be the sole metabolic link between PABA and methionine synthesis. An alternative explanation, however, arises from the observation (unpublished) that methionine (but not homocysteine) exerts a greater accelerating effect than B_{12} on the growth of wild type in minimal medium; PABA does not accelerate it at all. These facts indicate that the rate of growth of *E. coli* can be limited by some reaction in the methylation of homocysteine that does not necessarily involve PABA or B_{12}.

Strehler (1950) has described a Neurospora mutant that grows on either PABA or methionine. The PABA-sparing action of methionine observed in *E. coli* suggests that this Neurospora strain may simply have an incomplete block, resulting in a limited synthesis of PABA that would permit growth when supplemented by methionine. A mutant of *E. coli* with similar characteristics (strain 1861-460) has been described by Lampen *et al.* (1949).

The failure of dimethylbenzimidazole and 1,2-dimethyl-4,5-diaminobenzene to spare PABA does not exclude these compounds as precursors of B_{12} in *E. coli*. These data will have greater significance if the benzene ring of B_{12} can be definitely shown to arise from PABA.

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SUMMARY

Participation of PABA in the synthesis of vitamin B_{12} is shown by the sparing effect of B_{12} on the PABA requirement of *Escherichia coli* mutants. Since the molar requirement of *E. coli* for B_{12} is only 1/50 its requirement for PABA, the latter compound might conceivably participate in B_{12} synthesis either as a catalyst or as a precursor. While a catalytic function is not excluded, structural origin of the benzene ring of B_{12} from PABA would readily explain the fact that certain mutants blocked early in the synthesis of aromatic metabolites do not require B_{12}, for the quintuple supplement required by these strains already contains PABA.

Methionine and B_{12} spare PABA to a similar extent, paralleling their equivalence as sulfonamide antagonists and as growth factors in certain auxotrophs. The relation of PABA to methionine synthesis therefore appears to depend on

the role of PABA in the formation of B_{12}, which in turn takes part in the formation of methionine from homocysteine.

Pteroylglutamic acid does not serve as a substitute for PABA in *E. coli*, even in the presence of B_{12}; citrovorum factor is also inactive. We cannot confirm the reported ability of PGA to replace thymine as a partial substitute for PABA.

Quantitative demonstration of sparing action was facilitated by cultivating the inocula in the presence of a limiting amount of PABA, thereby avoiding intracellular storage of this compound.

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