한국농화학회지 제 38권 제 1호(1995)
Agricultural Chemistry and Biotechnology
Vol. 38, No. 1, pp. 31~36(1995)

Effects of Feeding Intermediate and Starter Units on *Monascus* Pigments Production

Young-Ju Hong, Jeong-Gu Kim, Hyun-Chul Woo and Soo-Un Kim*

Department of Agricultural Chemistry and The Research Center for New Biomaterials in Agriculture, Seoul National University, Suwon 441-744, Korea

Abstract : To investigate the mechanism for the main chain-elongation process and the possibility of putative precursors as stater units in the biosynthesis of the *Monascus* pigments, feeding experiments with possible poly-β-ketide intermediates were carried out. Both crotonic acid and sorbic acid, especially in low concentrations, enhanced the pigment production while not increasing the dried mycelium weight appreciably. Also, it was observed that the feeding of sorbic acid and its ethyl ester was about two folds efficient in the pigment production than the feeding of crotonic acid and its ethyl ester. In addition to these acids, cinnamic acid and vinylacrylic acid were examined for their possibility as starter units. It was observed that the color of the culture fed with cinnamic acid was dominantly dark-red, but with overall decrease in the pigment production. When its ethyl ester was administered to the culture, however, the pigment production increased significantly. Also noted in 2D TLC study of the pigments was the increased production of red pigment and the formation of new red pigments(Recevied January 7, 1995; accepted February 18, 1995).

Introduction

Historically, *Monascus* have been used in the production of red rice wine and red soybean cheese in Asian countries.[1,2] In 1895, the fungus *Monascus purpureus* Went was identified as the organism responsible for the color of Red Rice(Ang Khak, 紅麴), a native preparation used as a food colorant and alcoholic beverages in parts of Java and China.[3] Yellow pigments monascin and monascoflavin, orange pigments monascorubrin and rubropunctatin, and red pigments monascorubramine and rubropunctamine have been isolated from *Monascus*.[1,3,4]

Birch and his colleagues[5,6] and a Japanese group[7] showed that the pigments is of β-ketide chain origin through extensive labelling experiments. Hadfield *et al.*[8] in 1967 showed that the β-oxo-lactone system of sclerotiorin, rotiorin, rubropunctatin, monascin and monascorubrin was derived from fatty acid, which was biosynthesized independently from the main chromophore. However, question still remains as to what biosynthetic stage β-oxo-lactone system is assembled into the main chain.

Although the general outlines of the *Monascus* pigment biosynthesis are by now well accepted, the details of the key chain-elongation process by which the poly-β-ketide carbon skeleton is assembled are still rather obscure. If the poly-β-ketide chain formation of the *Monascus* pigments resembles the biosynthesis of fatty acid, the condensation product of acetate and malonate would be converted into crotonate before the next chain-extending reaction. Otherwise, acetate units would be assembled into the poly-β-ketide intermediate by a series of condensation, and then the resulting intermediates processed to the main chromophore by an unspecified sequence of reduction and dehydration reactions to give the desired structure. Recent molecular genetic studies, however, favor the latter view.[9]

In this study, through the precursor feeding experiments, the possibility of the chain-elongation intermediate as starter units and the probable mechanism for the main chain-elongation process in the *Monascus* pigment biosynthesis were examined. Specifically, incorporation of crotonic acid and sorbic acid and the putative intermediates of the main chain-elongation process in the *Monascus* pigment biosynthesis was examined. Also studied was the possible incorporation of unnatural starter unit for the pigment production.

Materials and Methods

Reagents

Most solvents were purified before use. Benzaldehyde was purified through washing with aqueous 10% sodium carbonate solution, drying over anhydrous $MgSO_4$ and distilling by reduced pressure under the blanket of nitrogen. Diethyl ether and 1,4-dioxane were purified through sodium-benzophenone ketyl method. Sorbic acid

Key words : *Monascus* pigment, poly-β-ketide
*Corresponding author

was obtained from Showa Co.

Microorganisms and cultural medium

The *Monascus anka*(IFO4478) culture was obtained from Dr. Hyun-Soo Kim, Foods Research and Development Center, Cheil Sugar Co. The culture was spread on the slants containing medium C[10] and incubated at 30℃ until the fungi had conidiated. The slant was treated with 50% glycerol solution and kept at −20℃. The strains U90-33 and U90-34 having high productivity of pigments were screened from the UV-induced mutants of the strain N3 (wild type) which was screened from the parent strain of *Monascus anka*.[11] The strains were used in further experiments for 1 year without remarkable reversion. The following standard cultivation protocol was used for all the experiments, unless indicated otherwise: aliquots (2~3 m*l*) of the conidial suspension were inoculated into Lin's medium[12] (100 m*l* per 500 m*l* Erlenmeyer flask) and incubated at 29~30℃ on a shaking incubator(150 rpm). After 2 days, aliquots(1 m*l*) of Lin's medium were transferred into Nishikawa's medium[13] (10 m*l* per petri dish) and incubated at 30℃ for 15 days.

Feeding experiments

Precursors were fed to the culture on the fifth day after inoculation as follows. The culture in Nishikawa's medium was washed with 5 m*l* of sterilized water. Each precursor dissolved in 1 m*l* of absolute ethyl alcohol was added to the culture with 10 m*l* of Nishikawa's medium containing 5% sucrose. On the tenth day after feeding, the cultures were harvested, and the pigments were determined. For the pulse-feeding experiment, the precursor was pulse-fed in two portions. Each addition was done by displacing the medium with 10 m*l* of the fresh Nishikawa's medium modified to 1% sucrose containing half of the precursor dissolved in 0.5 m*l* of absolute ethyl alcohol. The second addition of the precursor was made on the fifth day after the initial feeding and the culture was harvested five days later. The pigments were determined with UV-visible spectrophotometer. The pigment extract was then concentrated and two dimensional thin layer chromatography (TLC) was carried out for further analysis in the solvent systems PAA and HDA(*vide infra*).

General methods

TLC separations were carried out on pre-coated silica gel plates with fluorescence indicator(0.25 mm thickness, Sigma) and preparative TLC separations were on pre-coated silica gel plates without fluorescence indicator(0.25 mm thickness, Sigma) which had been washed with MeOH and dried at 120℃ in an oven for 3 hours. The TLC separations were performed with the solvent system PAA, petroleum ether/acetone/acetic acid (21:18:1) and then the solvent system HDA, n-haxane/1,4-dioxane/acetic acid (20:10:1).

Determination of pigments

The culture from Nishikawa's medium was filtered through filter paper(Whatman filter paper, No.2) and washed with distilled water. The mycelia were lyophilized with a freeze-dryer. The dry mycelia were ground in a mortar and the resulting powder was extracted by shaking for 48 hours with 20 m*l* of chloroform. After the pigment extract was filtered through glass wool, aliquots(10~20 μ*l*) of each extract were diluted with chloroform and examined with a UV-visible spectrophotometer[Hewlett Packard 8452A Diode-Array Spectrophotometer(Germany)]. Each production of yellow, orange and red pigment was estimated from absorbance at 392, 466 and 536 nm, respectively. Values of λ_{max} for each pigment was obtained by the following method. The pigment extract was concentrated, dissolved in chloroform and spread as a band on TLC plates. The first separation was performed in the solvent system PAA. The band corresponding to each pigment was scraped off, eluted with chloroform. The second separation was performed in the solvent system HDA.

Synthesis of precursor

Crotonic Acid: Fifty six microliters of acetaldehyde(1.0 mmole) was mixed with the reaction solution consisting 0.0521 g of malonic acid(0.5 mmole) and 0.1 m*l* of dry pyridine under the stream of nitrogen. The mixture was left in a refrigerator for 24 hours and warmed in a water bath(40~50℃) until the evoution of carbon dioxide had ceased. The mixture was then cooled in an ice bath, acidified by the addition of 53 μ*l* of 1:1 sulfuric acid and left in the refrigerator for 3~4 hours. The crude crotonic acid was extracted with three 1.0 m*l* portions of diethyl ether. The ethereal exract was dried over anhydrous $MgSO_4$, and the ether was evaporated. The crude acid collected was purified by preparative TLC in the solvent system, acetone/n-hexane (1:1). ^{1}H-NMR spectrum showed the product has *E* configuration as judged by the typical *J* values of 15.5 Hz between trans H's and 1.6 Hz between cis CH_3 and H.

Vinylacrylic Acid: Sixty six microliters of acrolein(1.0 mmole) was mixed with 1 m*l* of the reaction solution, and the subsequent processes were same as described above. ^{1}H-NMR spectrum showed a signal at 7.4 ppm(dd, J=11.2 Hz, 10.7 Hz), typical of the *E* compound.

Cinnamic Acid: Seventy five microliters of benzaldehyde(0.75 mmoles) was mixed with 1 m*l* of the reaction solution described above, and refluxed in a water bath for 3 hours under the stream of nitrogen. The subse-

quent processes were same as in crotonic acid. ^{1}H-NMR specrum showed the J value of 15.9 Hz between two vinylic H's indicating E configuration.

Ethyl Esters: Ethyl crotonate, ethyl sorbate, ethyl cinnamate-were synthesized by the following method. Absolute ethanol(0.12 mole) was mixed with 0.025 mole of acid and 0.2 ml of concentrated sulfuric acid was added to the mixture. The mixture was refluxed for 12 hours in a stream of nitrogen and then cooled. To the mixture was added distilled water. Ethyl ester was extracted with three 20 ml portions of diethyl ether from the mixture. The ethereal extract was washed with 5% sodium carbonate solution until the effervescent ceased, dried over anhydrous $MgSO_4$, and then the ether was evaporated. ^{1}H-NMR spectra of these ethyl esters were consistent with the structure.

Results and Discussion

Optimization of feeding experiments

For the experiment with *M. anka*, high pigment producing strains were screened through UV mutation, and strains U90-33 and U90-34 were selected as described. The final dried micelium weights from the strains N3 (wild type) and U90-33 were 0.25 g and 0.40 g, respectively. Furthermore, the final orange pigment production of the latter strain, as determined by the UV-visible spectrum, was nine folds higher than that of the former strain. Therefore, the following feeding experiments were carried out with the strain U90-33. In a time course study, the pigment production started after 5 days of incubation on Nishikawa's medium and leveled off around 15th day of incubation. The optimum feeding and the harvesting schedule was thus set as the 5th day and the 15th day after the inoculation of Nishikawa's medium, respectively.

To investigate the optimum feeding concentration of the precursors, the pigment production was examined in varied concentrations. The putative precursors were fed in free acid form, and as ethyl esters to enhance the uptake of the precursors through the cell membrane.

The result of the experiment is summarized in Table 1. It was in low concentration that most precursors increased the pigment production. In the case of 0.01 mM of crotonic acid and sorbic acid, the pigment production increased by about 30~40% and 70~500%, respectively. Higher concentration of the precursor feeding tends to decrease the pigment production except in the case of crotonic acid. Feeding of the ethyl esters was more efficient in the pigment production than with the free acids. The extent of the increase was more pronounced for red pigment. Cinnamic acid did not cause the increase of pigment production. However, in case of the ethyl cinnamate, the pigment production increased except for the higher concentration. Vinylacrylic acid decreased the production of yellow and orange pigments. Through these results, it was concluded that the most economical concentration for the feeding of both free acid and ester was 0.01 mM, and that the esterification seemed to be effective except in the case of cinnamic acid.

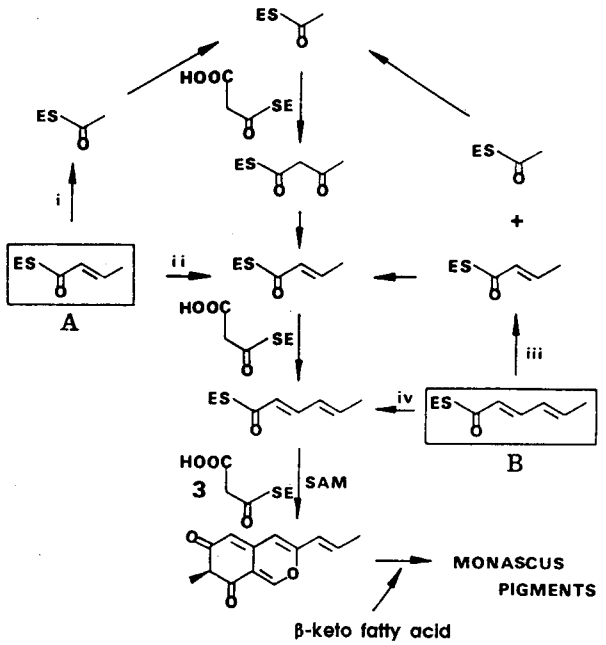
The pulse-feeding technique was employed to overcome concentration limit in the precursor feeding. Ten

Table 1. The effect of the putative precursors and the surrogate starter units on the *Monascus* pigment production. Values are net increase compared to those of the culture grown on Nishikawa's medium for 15 days and represent means of two replications.

Treatment	Increase (%)		
(mM)	A_{392}	A_{466}	A_{536}
Crotonic acid			
0.01	34.4	47.4	4.1
0.10	7.1	10.2	−7.7
1.00	15.4	24.7	265.6
Sorbic acid			
0.01	73.0	72.2	573.3
0.10	4.0	10.4	−3.6
1.00	7.7	10.5	42.6
Cinnamic acid			
0.01	−1.0	−0.1	−11.3
0.10	−6.7	−12.3	−26.7
1.00	−17.3	−21.9	−7.7
Vinylacrylic acid			
0.01	−13.2	−17.3	−6.8
0.10	−21.9	−26.4	12.0
1.00	−38.1	−49.0	20.5
Ethyl crotonate			
0.01	45.5	44.4	392.3
0.10	31.4	32.6	615.4
1.00	22.4	15.6	214.4
Ethyl sorbate			
0.01	36.4	40.2	261.5
0.10	83.8	89.4	607.7
1.00	40.1	33.5	346.2
Ethyl cinnamate			
0.01	51.1	44.2	461.5
0.10	28.7	7.2	573.3
1.00	−25.6	−40.8	311.8

Table 2. The effect of pulse feeding of crotonic acid on the pigment production. In the pulse-feeding experiment, two portions of 10 mol of crotonic acid were fed to the culture on the 5th and the 10th day after incubation as described in the Materials and Methods.

Treatment	Optical Density of $CHCl_3$ Extract		
	A_{392}	A_{466}	A_{536}
One-portion Feeding	36	23	3.1
Pulse Feeding	49	46	4.6



Scheme 1. Hypothetical process for the assembly of the putative precursors, crotonate(A) and sorbate(B), into the main chain of the *Monascus* pigments.

micromols of crotonic acid was fed in two egual portions at and 10th day after inoculation. It was found that the technique enabled the precursors to efficiently increase the pigment production. In the case of the pulse-feeding, the pigment production was about two folds higher than that of the one portion feeding(Table 2).

Possible mechanism for the main chain-elongation process in the *Monascus* pigment biosynthesis

To investigate the mechanism for the main chain-elongation process in the biosynthesis of the *Monascus* pigments, feeding experiments with possible poly-β-ketide intermediates were carried out. Both crotonic acid and sorbic acid, especially in low concentrations, enhanced the pigment production with dried mycelium weight not increasing appreciably; control 0.38 vs. 0.32~0.37 g/petri dish. It was observed that the feeding of sorbic acid and its ethyl ester was about two folds efficient in the pigment production than the feeding of crotonic acid and its ethyl ester, respectively (Table 1). A possible explanation of this is that the fed sorbate was converted to crotonate and acetate via fatty acid oxidation and the resulting crotonate and acetate participated in the chain-extending process of the pigments as shown in pathway iii (Scheme 1). Another possible pathway to contribute to the pigment biosynthesis is direct participation of sorbate in the biosynthesis as shown in pathway iv (Scheme 1).

Though some precursors could be degraded to acetate and incorporated into the pigments (i in Seheme 1), these results strongly support that crotonic acid and sorbic acid are the possible direct intermediates in the pigment biosynthesis (ii and iv in Scheme 1). Also, the above results are consistent with the processive backbone assembly mechanism.[9,14] In othe words, the main chain-elongation process of the *Monascus* pigment biosynthesis is analogous to that of the fatty acid biosynthesis where modification in chain structure follows after each chain elongation step.

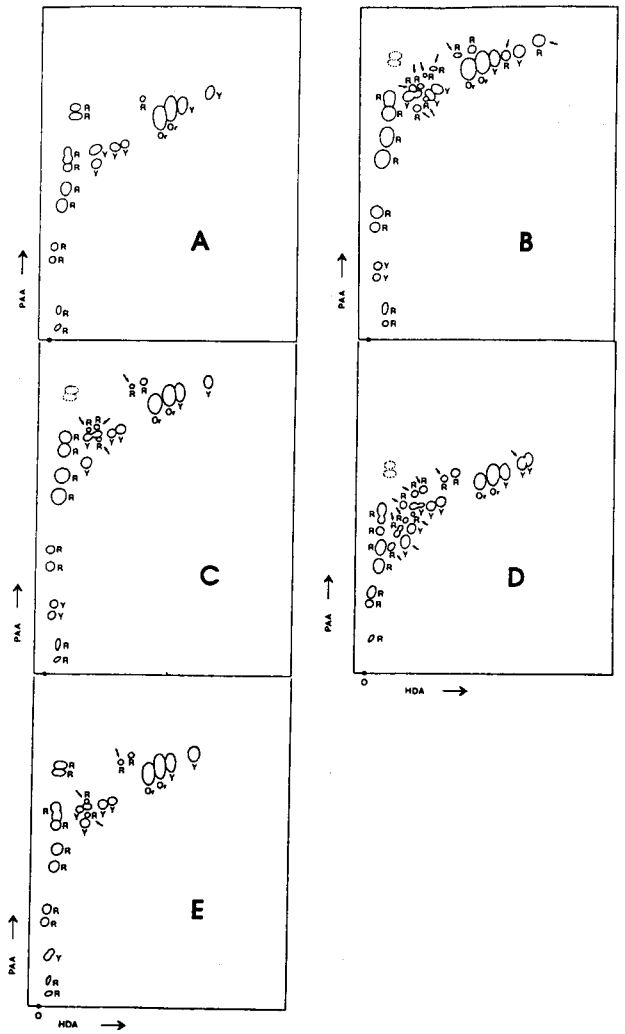


Fig. 1. 2D-TLC of the pigments isolated from *Monascus anka* U90-33 mycelia grown on Nishikawa's medium for 15 days. Putative new pigments are shown with arrows and the pigments disappeared on precursor feeding were indicated with broken line. Y stands for yellow, R red and Or orange. A, no precursor feeding; B, fed with vinylacrylic acid; C, fed with crotonic acid; D, fed with sorbic acid; E, fed with cinnamic acid.

Possible starter Units of *Monascus* pigment biosynthesis

The possible participation of crotonic acid and sorbic acid as starter units of the poly-β-ketide chain in the *Monascus* pigment biosynthesis was investigated. It was shown in the previous section that both crotonic acid

and sorbic acid were possible starter units in the *Monascus* pigment production. In addition to these acids, cinnamic acid and vinylacrylic acid were examined for the possibility as starter unit. It was observed that the color of the culture fed with cinnamic acid was dominantly dark-red, but with overall decrease in pigment production (Table 1). When its ethyl ester was administered to the culture, however, the pigment production increased, notably in the production of the red pigment. Furthermore, vinylacrylic acid, especially in high concentration, selectively increased the production of the red pigments. In the case of 1.00mM vinylacrylic acid feeding, the production of red pigments increased by 20%, while the production of yellow and orange pigments decreased remarkably (Table 1). From these results, one can assume that the feeding of cinnamic and vinylacrylic acids caused the formation of new pigment(s) which have more conjugations in the main chromophore to give more red characteristics. It is possible that sorbate, condensation product of crotonic acid and malonic acid, occasionally serves not only as chain-elongation intermediate but also as an abnormal starter unit of the *Monascus* pigment biosynthesis when fed in high concentration. Nevertheless, it is still possible that feeding of these compound caused enhanced reaction of the orange pigments with amines to from red ones.

Two dimensional TLC of the pigments obtained from the cultures fed with putative precursors indicated the formation of new pigment(s): several new red spots were observed on 2D TLC of the pigments isolated from the cultures fed with sorbic, cinnamic and vinylacrylic acids with disappearance of the two red pigments (Fig. 1). However, in the case of crotonic acid, a possible normal chain-elongation intermediate and a possible normal starter unit in the *Monascus* pigment biosynthesis, the original pigments were still observed. Furthermore, although the distribution of the pigments from the culture fed with crotonic acid were mostly consistent with that from the normal culture, several additional new red spots were found. These observations are highly consistent with the present finding with absorbance pattern.

It is now possible that crotonic, sorbic, cinnamic and vinylacrylic acids could serve as abnormal starter units and caused the formation of new pigments in the *Monascus* pigment biosynthesis. Further support for the incorporation presented above came from the finding that most new red pigments had considerably higher Rf value than those of the conventional red pigments in the solvent system HDA. Since new pigment(s) have more conjugations than yellow pigments, the pigments should possess larger λ_{max} values, and would behave more like yellow or orange pigments in 2D-TLC than the red pigments with amino functional group which would lower R_f-values in HDA.

These results, if interpreted as above, have provided important implications for the flexibility of the *Monascus* pigment biosynthesis to give a possible production of new pigments by feeding noble starter units. To confirm the incorporation of the precursors into the pigments, feeding experiment with labeled compound and structural determination of the putative new pigments would be necessary. Nevertheless, the feeding of unnatural starter units can be exploited to produce novel *Monascus* pigments for specialty use.

Acknowledgements

This work was supported by the grant from Korea Science and Engineering Foundation. YJH and JGK thank fellowship from The Research Center for New Biomaterials in Agriculture, Seoul National University.

References

1. For review, 蘇遠志 (1974) 紅麴菌の性質及びその利用について, 醱酵協會誌 **33**, 28-36.
2. For review, 遠藤章 (1985) 紅麴と紅麴菌をめでる歴史お最近の動向, 醱酵と工業 **43**, 544-552.
3. Went, F. A. F. C. (1895) *Monascus purpureus*, le champignon de l'Ang-Quac, une nouvelle Thelebolee. *Ann. Sci. Nat. Bot. Ser.* VIII **1**, 1-18.
4. For review, Su, Y. C. (1983) Fermentative production of Anka-pigments(*Monascus*-pigments). *Kor. J. Appl. Microbiol. Bioeng.* **11**, 325-337.
5. Birch, A. J., A. Casser, P. Fitton, J. S. E. Holer, H. Smith, G. A. Thompson and W. B. Whalley (1962) Studies in relation to biosynthesis. Part XXX. Rotiorin, monascin, and rubropunctatin. *J. Chem. Soc.* 3583-3586.
6. Holer, J. S. E., J. Staunton and W. B. Whalley (1964) The biosynthesis of fungal metabolites. Part I. Two different pathways to β-ketide chains in rotiorin. *J. Chem. Soc.* 16-22.
7. Kurono, M., K. Nakanishi, K. Shindo and M. Tada (1963) Biosynthesis of monascorubrin and monascoflavin. *Chem. Pharm. Bull.* **11**, 359-362.
8. Hadfield, J. R., J. S. E. Holker, D. N. Stanway (1967) The biosynthesis of fungal metabolites. Part II. The β-oxo-lactone equivalents in rubropunctatin and monascorubrin. *J. Chem. Soc.*(C) 751-755.
9. For review, Hopwood, D. A. and D. H. Sherman (1990) Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Ann. Rev. Genetics* **24**, 36-66.
10. Hiroi, T., T. Shima, T. Suzuki, M. Tsukioka and N. Ogasawara (1979) Hyperpigment-productive mutant of *Monascus anka* for solid culture. *Agric. Biol. Chem.* **43**, 1975-1976.
11. Kim, S.-U., and J.-G. Kim (1990) Pigment production in *Monascus anka*. *J. Kor. Agr. Chem. Soc.* **33**, 239-246.
12. Lin, C. F. (1973) Isolation and cultural conditions of *Monas-*

cus sp. for the production of pigment in a submerged culture. *J. Ferment. Technol.* **51**, 407-414.
13. 西川英次郎 (1932) 絲狀菌の生化學 (其の一) 紅麴菌 (*Monascus purpureus* Went)の色素 (第一報), 日本農藝化學會誌 **8**, 1007-1015.
14. Donadio, S., M. J. Staver, J. B. McAlpine, S. J. Swanson and L. Katz (1991) Modular organization of genes required for complex polyketide biosynthesis. *Science* **252**, 675-679.

색소 중간체와 개시체 투여가 Monascus 색소생산에 미치는 영향

홍영주, 김정구, 우현철, 김수언*(서울대학교 농화학과 및 농업생물신소재 연구센터, 경기도 수원시 서둔동 103)

초록 : *Monascus*색소 주탄소골격의 생성 메카니즘을 연구하고, 새로운 개시체가 색소 생합성에 미치는 영향을 연구하고자 적절한 중간체와 개시체를 투여하였다. 크로톤산과 소르브산은 낮은 농도에서 균체의 증가없이 색소 형성을 촉진하였다. 소르브산이나 그의 에틸에스테르를 투여하였을 때에는 크로톤산의 경우보다 약 2배 색소형성이 많았다. 이외에도 신남산과 비닐아크릴산을 개시체로 투여하였을 때에는 색소 혼합물은 암적색으로 나타났으며 색소의 형성은 감소하였다. 그러나 에틸에스테르로 변환시켜 투여하였을 때에는 색소의 형성이 증가하였으며 이차원 TLC 결과 새로운 적색소가 생성되었음을 알 수 있었다.

*연락저자