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ORIGINAL PAPER

Optimization of Synthetic Conditions for the Preparation of Dihomo-γ-Linolenic Acid from γ-Linolenic Acid

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Abstract Orthogonal experiments were employed to optimize the correlated parameters of reduction, sulfonation, substitution and hydrolysis. These reactions were used to convert γ-linolenic acids into dihomo-γ-linolenic acids (DGLA). For the reduction, the best reaction conditions were at 35 °C for 4.5 h with $LiAlH_4$ and γ-linolenic acid (in the ratio of 40 g:100 g); for the sulfonation, reaction at 29 °C for 3.5 h with 150 g γ-linolenic alcohol and 65 mL mesyl chloride, then the water phase being extracted with dichloromethane (3 × 100 mL); for the substitution, the reaction at 80 °C for 2.5 h with metallic sodium and sulfonate (at a ratio of 8 g:100 g); and for the hydrolysis, reaction at 80 °C for 2.5 h with NaOH and dihomo dioate (at a ratio of 50 g:100 g). The four reactions gave yields that exceeded 90% for each step. Finally, crystallization and decarboxylation provided DGLA in an overall yield of 60% and >95% purity.

Keywords Dihomo-γ-linolenic acid (DGLA) · Reduction · Sulfonation · Substitution · Hydrolysis · Crystallization and decarboxylation reaction · Orthogonal experiment

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Abbreviations

AA	Arachidonic acid
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexenoic acids
EPA	Eicosapentaenoic acid
GLA	γ-Linolenic acid
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
I	γ-Linolenic alcohol
II	γ-Linolenic sulfonate
III	Dihomo dioate
IV	Dihomo dioic acid

Introduction

Lipids supply most of the nonglucose fuel calories and they are building blocks for cellular components and essential fatty acids [1]. Long-chain polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA), arachidonic acid (AA), docosahexenoic acids (DHA) and dihomo-γ-linolenic acid (DGLA), mainly prevalent in fish oils, have been demonstrated to play important roles in human health and nutrition [2].

DGLA is a precursor for the biosynthesis of prostaglandin E_1 [3, 4]. It has been reported that DGLA and DGLA-containing oil (triacylglycerol) might inhibit the activity of thromboxane A_2 [5], suppress human platelet aggregation and mitogen release, and exert anti-inflammatory [6], antihypertensive [7], antiatherosclerotic, and antiallergic effects [8, 9]. In the human body, these essential and immunoregulatory long-chain omega-6 fatty acids are only available from dietary sources [10, 11]. As a precursor to the synthesis of DGLA, γ-linolenic acid (GLA,

18:3n-6) is a key molecule participating in omega-6 fatty acid metabolism which leads to the rapid production of longer chain omega-6 fatty acids such as DGLA (20:3n-6), AA (20:4n-6), PGE_1 and PGE_2, etc. [12].

It has also been reported that DGLA could be synthesized by microbes [13, 14], but the content of DGLA in hyphae was too low to make it a precursor for the synthesis of PGE_1. Sun Qiliang reported that DGLA could be synthesized chemically [15] with the purity of DGLA ranged from 80 to 90% and a yield of 35 to 42%. And Hong et al. also reported an improved method for the preparation of γ-linolenic acid and synthesis of dihomo-γ-linolenic acid, in which they used complex-sodium boron hydride to reduce the methyl ester of γ-linolenic acid, and then to prepare dihomo-γ-linolenic acid by methyl sulfonyl chloride-malonic ester synthesis with a purity of 66% [16].

Base on previous research, in this study, an orthogonal experiment was used to find the optimum correlated parameters for reduction, sulfonation, substitution and hydrolysis reactions in the synthesis of DGLA, such as the reaction time, temperature and so on, and synthesize DGLA with a purity exceeding 95% and an overall yield of 60% under these optimized conditions.

Experimental Procedures

Materials

98% GLA was produced in our laboratory. It is isolated from evening primrose oil by urea inclusion crystallization [17]. $LiAlH_4$, 2,4,6-trimethylpyridine and mesyl chloride were purchased from China Fluka company. The standard sample of DGLA was purchased from China Jilin Provincial Institute for Drug Control. All chemicals and solvents used were of analytical grade. Distilled and deionized water was used throughout the whole experiment.

Gas Chromatography Analysis of GLA and DGLA

A mixture sample containing 0.1 g DGLA or GLA and 2 mL 0.5 M solution of KOH in methanol was heated at 63 °C for 20 min with stirring, and 0.2 mL solution of BF_3 in diethyl ether was then added. After heating for 5 min, the mixture was cooled and extracted by using 2 mL petroleum ether from which 2 μL of the upper solution was taken out as the sample. Then the GC-14B gas chromatography (HITACHI) set using a capillary column (INNOWAX 19095 N-123) was heated. When the temperatures of the column, detector and injector of the set reached 230, 280 and 250 °C, respectively, the sample was injected into the GC set. In the process of analysis, the nitrogen carrier gas flow rate was set at 15 mL/min, the air flow rate at 400 mL/min and the hydrogen flow rate at 50 mL/min. The purity of GLA or DGLA was calculated by the integral area normalization method.

Typical Procedure for the Preparation of DGLA

Figure 1 shows a typical procedure for synthesizing DGLA from GLA in a six-step reaction sequence.

Reduction

The reduction of GLA by $LiAlH_4$ was carried out in a three-neck flask. First, 60 g $LiAlH_4$ (~1.5 mol/L) was added to 2,750 mL diethyl ether to form a mixture which was heated up to 35 °C, stirred for 1–1.5 h, and then cooled below 25 °C, 150 g GLA (~0.55 mol/L) was added dropwise into the flask with stirring and aerating N_2 as a protective gas, and after the mixture was raised to 35 °C for refluxing 4–4.5 h, the flask was cooled down below 10 °C in an ice water bath and 250 mL ice water was added; finally, the resultant **I** was extracted with diethyl ether (3 × 100 mL) and following the evaporation of diethyl ether, **I** was obtained.

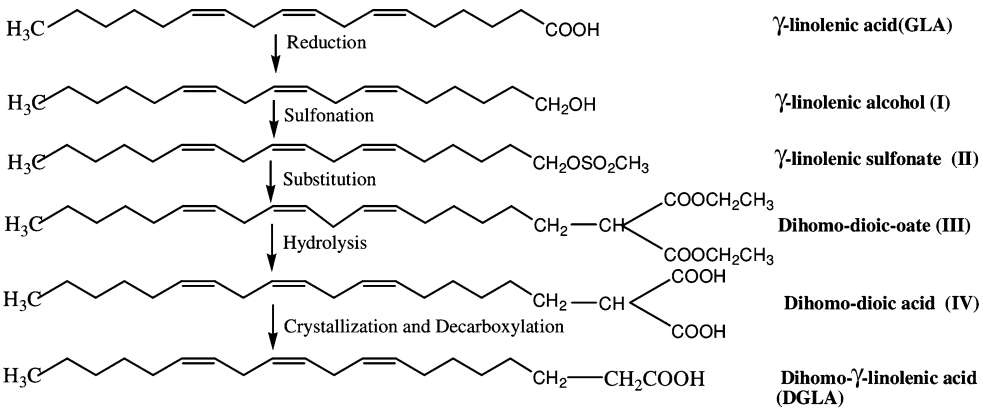

Fig. 1 Synthesis of DGLA from GLA

Sulfonation

A 470-mL amount of dichloromethane, 150 g **I** (~0.57 mol/L) and 86 mL 2,4,6-trimethylpyridine (~0.7 mol/L) were added one after the other to a 3-L three-neck flask. The flask was cooled down in the ice water bath to below 2 °C and then 65 mL mesyl chloride (~0.55 mol/L)was added. The reaction was performed at 26–29 °C for 4–4.5 h with stirring and aerating with N_2 as a protective gas. After that, the temperature of the flask was cooled down to 4 °C, and 300 mL distilled water was added. Next, 4 mol/L HCl was used to adjust the pH value of the solution to about 1–2. The dichloromethane phase was isolated and the water phase was extracted with dichloromethane (3 × 100 mL). The extraction method was designed at three levels (C_1 means Method 1, in which, the dichloromethane phase containing **II** was washed five times with 1 mol/L HCl, and all the aqueous phases were discarded. After dichloromethane was evaporated, **II** was obtained. C_2 means Method 2, in which, the process was repeated as in Method 1 except that the aqueous phases derived from the first and second washing were extracted with dichloromethane. And C_3 means Method 3. The process was similar to Method 1, but the aqueous phases derived from the first to the third washing were extracted by using dichloromethane). The dichloromethane phases were combined and washed to neutrality with 1 mol/L HCl and water. Following the evaporation of dichloromethane, **II** was obtained.

Substitution

III was prepared by dissolving 22 g metallic sodium (~1 mol/L) in 1,050 mL dehydrated alcohol below 18 °C for 15 min with stirring and aerating N_2 as a protective gas and then adding 240 mL diethyl malonate (~1.5 mol/L) and refluxing at 80 °C for 1–1.5 h. Next, the temperature of the solution was cooled to 30–40 °°C and 195 g sulfonate (~0.6 mol/L) was added. After the mixture was refluxed at 80 °C for 2 h, the temperature of the flask was dropped to 50 °C and 700 mL water was added. 4 mol/L HCl was used to adjust the pH value of the mixture to 1–2, the oil phase was separated, and the water phase was extracted with petroleum ether (3 × 100 mL). Finally, all the petroleum ether phases containing oil were combined and washed by water until a pH value of 5–6. Following the evaporation of the petroleum ether, **III** was obtained.

Hydrolysis

A mixture containing 220 g **III** (~0.54 mol/L) and 95 g NaOH (~0.42 mol/L) in 870 mL water and 1,800 mL ethanol was hydrolyzed at 80 °C for refluxing for 2 h with stirring and aerating with N_2 as a protective gas. Then the temperature of the mixture was lowered to 50 °C and the oil phase separated out after the adjustment of the pH value to about 2–3 by 6 mol/L H_2SO_4. The resultant water phase was extracted with diethyl ether. Then all the diethyl ether phases containing oil were combined and washed with water until a pH value of about 6 was obtained. Following the evaporation of the diethyl ether, **IV** was obtained.

The yield of the products obtained from the above procedures was calculated as follows:

$$\text{The yield} = \frac{\text{The actual weight of the product (g)}}{\text{The theoretical weight of the product (g)}} \times 100\% \quad (1)$$

Crystallization and Decarboxylation

The petroleum ether was added to **IV** in the volume ratio of 3:1, then the mixture was cooled to −20 °C for 24 h and the impurities of the crystallized products were removed by washing with cool petroleum ether. Decarboxylation at 150–160 °C for 3 h in an oil bath under vacuum gave DGLA. The calculation of the yield from GLA to DGLA was expressed as follows:

$$\text{The yield} = \frac{\text{The actual weight of DGLA (g)} \times \text{its purity (\%)}}{\text{The weight of GLA (g)} \times \text{its purity (\%)}} \times 100\% \quad (2)$$

Statistical Analysis [18]

Statistical evaluations of the differences among the different treatments were calculated by the analysis of variance software (ANOVA) with SPSS10.0 (Statistical Program for Social Sciences), Chinese Version. Differences were considered significant at the 5 and 1% levels.

Results and Discussion

The Reduction of GLA

An $L_9(3^4)$ orthogonal experiment [19] was employed for optimizing such technical parameters such as factor *A*-reaction time ($A_1 = 3.5$ h, $A_2 = 4$ h, $A_3 = 4.5$ h), factor B-reaction temperature ($B_1 = 25$ °C, $B_2 = 30$ °C, $B_3 = 35$ °C), and factor *C*-the amount of $LiAlH_4$ added to per 100 g GLA ($C_1 = 40$ g, $C_2 = 50$ g, $C_3 = 60$ g). The results shown in Table 1 indicate that the optimal sequence and combination were *B–A–C* and $A_3B_3C_3$, respectively, by the range analysis. The results of variance analysis are listed in Table 2 which indicates that the effect of factors A and B on the yield revealed a significance at 5% level.

Table 1 The results of orthogonal experiment for reduction, sulfonation, substitution and hydrolytic reactions

No.	Factor				Yield			
	A[a]	B[b]	C[c]	D[d]	E[e]	F[f]	G[g]	H[h]
1	1	1	1	1	67.58	90.87	91.23	86.24
2	1	2	2	2	85.21	95.55	94.06	89.25
3	1	3	3	3	91.22	97.78	94.28	89.67
4	2	1	2	3	75.36	92.45	95.71	91.04
5	2	2	3	1	95.37	97.45	96.33	92.33
6	2	3	1	2	96.56	93.36	93.31	89.25
7	3	1	3	2	86.25	95.89	95.54	91.03
8	3	2	1	3	95.89	93.66	93.59	90.22
9	3	3	2	1	98.22	96.75	96.75	92.33
k_1	81.34	76.40	86.68		Reduction			
k_2	89.10	92.16	86.26		Optimal sequence: *B–A–C*			
k_3	93.45	95.33	90.95		Optimal combination: $A_3B_3C_3$			
k_1	94.73	93.07	92.63		Sulfonation			
k_2	94.42	95.55	94.92		Optimal sequence: C–B–A			
k_3	95.43	95.96	97.04		Optimal combination: $A_3B_3C_3$			
k_1	93.19	94.16	92.71		Substitution			
k_2	95.12	94.66	95.51		Optimal sequence: C–A–B			
k_3	95.29	94.78	95.38		Optimal combination: $A_3B_2C_2$			
k_1	88.39	89.44	88.57		Hydrolysis			
k_2	90.87	90.60	90.84		Optimal sequence: *A–C–B*			
k_3	91.16	90.42	91.01		Optimal combination: $A_3B_2C_3$			

[a–d] Factor *A*, *B*, *C*, and *D* (black). For reduction (*A*-reaction time, *B*-reaction temperature, *C*-the amount of $LiAlH_4$ added to per 100 g GLA); For sulfonation (*A*-reaction time, *B*-reaction temperature, *C*-extraction method); For substitution (*A*-reaction time, *B*-reaction temperature, *C*-the amount of added metallic sodium); For hydrolysis (*A*-reaction time, *B*-reaction temperature, *C*-the amount of NaOH added to per 100 g **III**)

[e–f] The yield of reduction, sulfonation, substitution and hydrolytic reaction products

Therefore, factors A and B should be controlled at the optimal level and factor C held at any level, i.e. $A_3B_3C_1$. With this combination, the experiment was performed at 35 °C for 4.5 h with 40 g $LiAlH_4$ added into per 100 g GLA. The yield of I exceeded 90%.

The final products of the reduction might include **I**, LiOH, Al $(OH)_3$, $LiAlO_2$, which were the proportional to GLA and $LiAlH_4$. A sediment of $Al(OH)_3$ produced in the reduction was granular but amorphous. When the product **I** was directly extracted with diethyl ether, the yield obtained was rather low, because **I** could be included in the sediment, similar to a urea inclusion complex. Thus, in order to destroy the crust of the inclusion, it was necessary to wash the sediment repeatedly with water till **I** was released. Then **I** was able to be extracted with diethyl ether from the water phase.

Sulfonation of the Product **I**

The influences of the reaction time, temperature and extraction method on the yield of sulfonation of the product I were investigated by employing an $L_9(3^4)$ orthogonal experiment, in which levels of the main factors were as follows: reaction time *A* ($A_1 = 3.5$ h, $A_2 = 4$ h, $A_3 = 4.5$ h), reaction temperature *B* ($B_1 = 23$ °C, $B_2 = 26$ °C, $B_3 = 29$ °C) and extraction method *C* (see the "Experimental Procedures"). The results of range analysis indicate that the optimal sequence of *C–B–A* and the optimal combination of $A_3B_3C_3$, and the results of the variance analysis, shown in Table 2, indicate that the effect of factors *B* and *C* on the yield revealed significant at 5 and 1% level, respectively. As factors *B* and *C* should be controlled at optimal level, therefore, the optimal combination was $A_1B_3C_3$. The experiment was performed at 29 °C for 3.5 h with Method 3. The yield of product **II** exceeded 95%.

Pyridine with strong basicity usually acted as an acid-binding agent. In sulfonation, 2,4,6-trimethylpyridine was added to absorb HCl. After the absorption, 2,4,6-trimethylpyridine was neutralized by adding excess HCl. During the experiment, when eluting the dichloromethane phase using 1 mol/L HCl, we found that the colors of the eluted aqueous phases from the first to the third time were all yellowish, which meant the aqueous phases had to be extracted with dichloromethane one more time.

Substitution of the Product **II**

For the substitution of the product **II**, the influences of the reaction time, temperature and amount of added metallic sodium on the yield were investigated by employing an $L_9(3^4)$ orthogonal experiment, in which reaction time is factor *A* ($A_1 = 1.5$ h, $A_2 = 2$ h, $A_3 = 2.5$ h), reaction temperature is factor *B* ($B_1 = 80$ °C, $B_2 = 85$ °C, $B_3 = 90$ °C), and for per 100 g **II**, the amount of added metallic sodium is factor *C* ($C_1 = 5$ g, $C_2 = 8$ g, $C_3 = 11$ g). The result of range analysis in Table 1 shows the optimal sequence was *C–A–B* and the optimal combination was $A_3B_2C_2$, and the variance analysis of Table 2 indicated the effect of factors *A* and *C* on the yield was significant at 5%. Therefore, factors *A* and *C* should be controlled at the optimal level and factor *B* may at any level, i.e. $A_3B_1C_2$. The experiment was carried out at 80 °C for 2.5 h with 8 g metallic sodium added per 100 g **II**. The yield of the **III** exceeded 95%.

For substitution, studies were made of the influences on the experimental results by the reaction time and temperature of both metallic sodium with ethanol and diethyl malonate with sodium ethoxide. At lower temperatures, metallic sodium took a long time to dissolve in ethanol. As soon as it was dissolved, if diethyl malonate

Table 2 The results of variance analysis

Source	*df*	SS	S^2	*F*	Sig.	*df*	SS	S^2	*F*	Sig.
Reduction						Sulfonation				
A	2	226.012	113.066	25.614*	0.038	2	1.615	0.808	6.338	0.136
B	2	617.066	308.533	69.631*	0.014	2	14.706	7.353	57.712*	0.017
C	2	40.337	20.169	4.571	0.179	2	29.185	14.593	114.533**	0.009
Error	2	8.824	4.412			2	0.255	0.127		
Total variance	8	892.239				8	45.762			
Substitution						Hydrolysis				
A	2	8.167	4.084	24.987*	0.038	2	14.163	7.082	33.214*	0.029
B	2	0.649	0.324	1.985	0.335	2	2.347	1.174	5.505	0.154
C	2	14.983	7.492	45.839*	0.021	2	11.278	5.639	26.447*	0.036
Error	2	0.327	0.163			2	0.426	0.213		
Total variance	8	24.126				8	28.215			

* Significant at the 5% level

** Significant at the 1% level

was added immediately and the temperature was raised simultaneously, a turbid or white precipitate was sure to appear, which meant the experiment had failed. The reason was probably that metallic sodium could not be completely used up in reacting with ethanol. When diethyl malonate was added, the residual sodium played the role of a catalytic agent and led to the failure of experiments. If there was N_2 present in the reaction system, the catalytic action of sodium could be accelerated. For this reason, diethyl malonate could not be added in the reaction system until the metallic sodium had completely reacted with the ethanol. When diethyl malonate was added below 20 °C for over 20 min, all the experiments were successful. Therefore, the reaction between sodium ethoxide and diethyl malonate should be below 20 °C for 20 min, and at the same time, the doses of sodium and diethyl malonate should be strictly controlled to avoid the white precipitate.

Hydrolysis of the Product **III**

An $L_9(3^4)$ orthogonal experiment was employed to optimize the technical parameters—factor *A*-reaction time ($A_1 = 1.5$ h, $A_2 = 2$ h, $A_3 = 2.5$ h), factor *B*-reaction temperature ($B_1 = 80$ °C, $B_2 = 85$ °C, $B_3 = 90$ 4C), and factor *C*-the amount of NaOH added to per 100 g **III** ($C_1 = 30$ g, $C_2 = 40$ g, $C_3 = 50$ g). The results, listed in Table 1, indicate that the optimal sequence was *A*–*C*–*B* and the optimal combination was $A_3B_2C_3$ by range analysis. And the results of variance analysis in Table 2 indicates that the effect of factors *A* and *C* on the yield were significant at the 5% level. Therefore, factors *A* and *C* should be controlled at the optimal level and factor *B* may at any level, i.e. $A_3B_1C_3$, thus the experiment was performed at 80 °C for 2.5 h with 50 g NaOH added to per 100 g **III**. The yield of product **IV** exceeded 90%.

The Effect of Crystallization Times and Separation Method on the Yield and Purity

Crystallization of **IV** was carried out at −10 °C for 24 h after petroleum ether was added to it at a volume ratio of 3:1. The crystallized products were able to be separated by vacuum filtration and washing with chilled petroleum ether (Method 1), or by centrifugation and washing with chilled petroleum ether (Method 2). The processes might have to be repeat several times. In each method, all the petroleum ether phases were combined and concentrated. Then new petroleum ether was added to the concentrated products in the volume ratio of 2:1, and the mixture was recrystallized three times in the same manner as mentioned above. Decarboxylation was carried out at 150 °C for 3 h under vacuum conditions. Following this step, DGLA was obtained.

The effects of the times and methods of crystallization on the purity and yield are shown in Table 3 and variance analysis indicated that the effects of the crystallization times and separation methods on the purity did not demonstrate significant at the 5% level, while the effects of the crystallization times and methods on the yield were significant at the 1% level. Therefore, in terms of the yields, variance analysis and Duncan's Test indicated that crystallization three times with Method 2 was the optimal choice.

In summary, from the results and analyses by $L_9(3^4)$ orthogonal experiment, we concluded that when all the optimal conditions mentioned above were adopted for synthesizing DGLA, the purity of DGLA could exceed 95% with an overall yield of 60%.

Table 3 The effects of the time and method of crystallization on the purity and yield

Crystallization times	Purity		Yield	
	Method 1 (B_1)	Method 2 (B_2)	Method 1 (B_1)	Method 2 (B_2)
One time (A_1)	95.89	94.67	46.36	58.64
	94.97	96.22	48.25	56.25
Two times (A_2)	96.04	96.11	53.44	63.43
	95.02	95.13	54.63	62.57
Three times (A_3)	95.63	95.37	55.25	65.37
	96.26	94.66	56.33	64.63

Purity of DGLA, yield using GLA as starting materials to synthesize DGLA

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