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Monopalmityloxy Shikimic Acid: Enzymatic Synthesis and Anticoagulation Activity Evaluation

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Abstract The monopalmityloxy shikimic acids have been synthesized from shikimic acid and palmitic acid catalyzed by Novozym 435 in 2-methyl-2-butanol. The anticoagulation activity *in vivo* via oral administration of monopalmityloxy shikimic acid has been evaluated through arteriovenous shunt model of rats and through the determination of thrombin time, prothrombin time, and activated partial thromboplastin time via rats. After reaction, the solid shikimic acid has been observed to dissolve in the reaction system completely. The subsequent high-performance liquid chromatography–mass spectroscopy analysis showed that the monopalmityloxy shikimic acids, as the only products, had been formed and the overall conversion rate was over 70%. The result showed that it had anti-thrombosis activity, could prolong the coagulating time and bleeding time *in vivo*, and lengthen the coagulating time *in vitro*. Compared with control group, the differences of the treatment group and aspirin group of rats are significant ($P<0.05$) for prothrombin time and thrombin time, and very significant ($P<0.01$) for activated partial thromboplastin time. It suggested that the product had the anticoagulation activity. The mechanism might be the co-action of the inhibition of intrinsic coagulation and the inhibition of extrinsic coagulation, and the inhibiting effect on intrinsic pathway is stronger than that on extrinsic pathway.

Keywords Monopalmityloxy shikimic acid · Shikimic acid · Novozym 435 · Anticoagulation · Anti-thrombosis activity

Introduction

Shikimic acid, (SA, $C_7H_{10}O_5$, R1–3=OH in Fig. 1) is a kind of bioactive natural product first isolated from the fruit of *Illicium religiosum* by Eykman in 1885 [1]. Ma et al. [2, 3] revealed that SA inhibited platelet aggregation and blood coagulation. SA inhibited rabbit platelet aggregation induced by ADP and collagen *in vitro* with IC_{50} of 9.25 and 3.56 mmol l^{-1},

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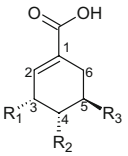


for SA R1-3=OH; logP=-0.92+/- 0.39
for TASA R1-3=$OCOCH_3$; logP=0.90+/- 0.4
for IPSA R1-2=cycle $OC(CH_3)_2O$; R3=OH; logP= 0.83+/- 0.45

Fig. 1 Structures of shikimic acid (*SA*), triacetyl shikimic acid (*TASA*), and isopropylidene shikimic acid (*IPSA*) and their corresponding calculated log P (calculated by ChemSketch)

respectively. SA 25 and 50 mg kg^{-1} i.p. for 3 days inhibited platelet aggregation induced by ADP in rats subjected to middle cerebral artery thrombosis. SA was shown to increase rat plasma 6-keto-$PGF_{1\alpha}$ and no significant effect on TXB_2 generation in PRP induced by ADP. SA 25, 50, and 100 mg kg^{-1} i.m. or i.v. elevated the blood coagulation time of mice. SA was also shown to have the antagonistic effects against focal cerebral ischemia injury in rats subjected to middle cerebral artery thrombosis [2]. SA can reduce the volume of cerebral infarction, decrease the neurological deficit scores, abate the brain edema, and can increase the cerebral blood flow in the ischemia area [2].

The poor bioavailability and blood–brain barrier (BBB) permeability correlated with strong polarity and hydrophilicity (solubility of SA in water is as high as 180 g l^{-1}), however, limited the general therapeutic utilization of SA. In order to increase its liposolubility and modify its log P, triacetyl shikimic acid [4–7] (TASA, R1–3=$OCOCH_3$ in Fig. 1) and isopropylidene–shikimic acid (IPSA, R1–2=cycle $OC(CH_3)_2O$, R3=OH in Fig. 1) [8–13] have been synthesized and evaluated for their physiological effects as anti-thrombosis agents and protective agents on brain damage induced by focal cerebral ischemia. Although the polarities of TASA and IPSA, compared with SA itself, have been modified and the corresponding calculated log P (calculated by ChemSketch) have been shifted from negative to positive, the values are still not big enough and there are no reports about their BBB permeability. In order to optimize the bioavailability, especially BBB permeability, it is strongly desired to design and synthesize a series of liposoluble derivatives of SA which allow one to adjust the lipophilicity (log P) flexibly.

In this paper, the biosynthesis system has been established and the monopalmityloxy shikimic acids (MPSA) have been successfully synthesized from shikimic acid and palmitic acid catalyzed by Novozym 435 in 2-methyl-2-butanol. After collection and purification, MPSA has been evaluated for its anticoagulation activity *in vivo* via oral administration (OS).

Materials and Methods

Novozym 435 (type B lipase from *Candida antarctica* immobilized on an acrylic resin) was a gift from Novo Nordisk (Denmark). According to the manufacturer, the preparation had an activity of 7,000 PLU/g (propyl laurate units) at 60°C.

The shikimic acid (purity >99%, HPLC) was purchased from Huateng Co. in Shaanxi Province. The purity of palmitic acid was over 98%. The purity of 2-methyl-2-butanol was more than 99%.

The tablet of acetyl salicylic acid was purchased from Wuxi Astra Co. Heparin, chloral hydrate, and the kits for determination of activated partial thromboplastin time, thrombin time, and prothrombin time were purchased from Shanghai Sun Biotech. Co.

IR: Nicolet Nexus FTIR spectrometer. HPLC-MS: Waters 2690 with Waters 996 detector, LichrosPHer C-18 2.6×250 mm column. Conditions: Waters Platform ZMD 4000 mass spectrometer. UV: UV-240 spectrometer from Shimadzu Corporation.

Clean ICR mice, half males and half females, aged 4 weeks and weighed 23–25 g, were obtained from Yangzhou University Medical Center [permit SCXK (Su) 2007-0001]. Clean SD rats, half males and half females, weighted 280–300 g, were obtained from Zhejiang Province Laboratory Animal Center [permit SCXK (Zhe) 2003-0001]

Enzymatic Synthesis, Purification, and Structure Identification of Monopalmityloxy Shikimic Acid

The reaction was carried out in vacuum rotary evaporator under 55°C, 0.1 kPa for 24–48 h, until the solid shikimic acid disappeared completely. Shikimic acid (0.1 mol), 5 g Novozym 435, and 200 ml solution of palmitic acid in 2-methyl-2-butanol (1 mol l^{-1}) were added to a 500-ml round bottom flask at the start of the reaction. Fresh solvent was added to maintain the volume of the reaction system constant. After the solid shikimic acid disappeared, the reaction was stopped and the mixture was filtered. The filtrate was then collected and evaporated to get the solid mixture. The residual solvents and water in the flask were dried by nitrogen.

The solid mixture, including monopalmityloxy shikimic acid, unreacted palmitic acid, and shikimic acid, were then purified by silica gel columns (particle size 50–75 μm). After sampling, the column were eluted by hexane (3V), ether (2V), $CHCl_3$/methanol/hexane (5:1:4) (2V), $CHCl_3$/methanol (5:1) (2V), and methanol (1V). The elution was collected separately and monitored by TLC [14]. The part containing product as the single component was collected and identified by high-performance liquid chromatography–mass spectroscopy (HPLC–MS).

For the HPLC, the column, LichrosPHer C-18 2.6×250 mm, was kept at 30°C. The mobile phase, 8% methanol–92% water (containing 1% acetic acid), was flowed at the rate of 0.3 ml min^{-1}. The sample with the volume of 5 μl was injected.

Single Dose Acute Oral Toxicity Test of Monopalmityloxy Shikimic Acid

After an adaptation period for 3 days, 20 mice were randomly divided into two groups of ten each. The dosage was 1,200 mg kg^{-1}. The mice in the treatment groups were orally

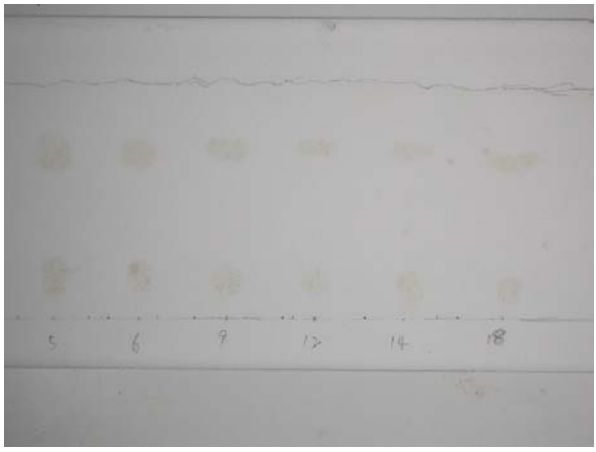


Fig. 2 Acylation of shikimic acid by C_5–C_{18} alkanoic acids analyzed by TLC

administered once, while the control group received the same volume (0.5 ml) of physiological saline. Then the mice were observed for 7 days and anatomized to observe the changes of viscera.

Determination of Prothrombin Time (PT), Thrombin Time (TT), and Activated Partial Thromboplastin Time (APTT)

After an adaptation period for 3 days, nine normal male SD rats were randomly divided into three groups of three each. The dosage was 100 mg kg^{-1}·day^{-1}. The MPSA was dispersed in water with the aid of Tween 10 (0.05 ml/10 ml). The rats in the treatment groups were orally administered continuously for 3 days, while the control group and aspirin group received the same volume of physiological saline and aspirin solution (300 mg kg^{-1} day^{-1}), respectively. One hour after the last administration, 4.5 ml of blood was taken from the

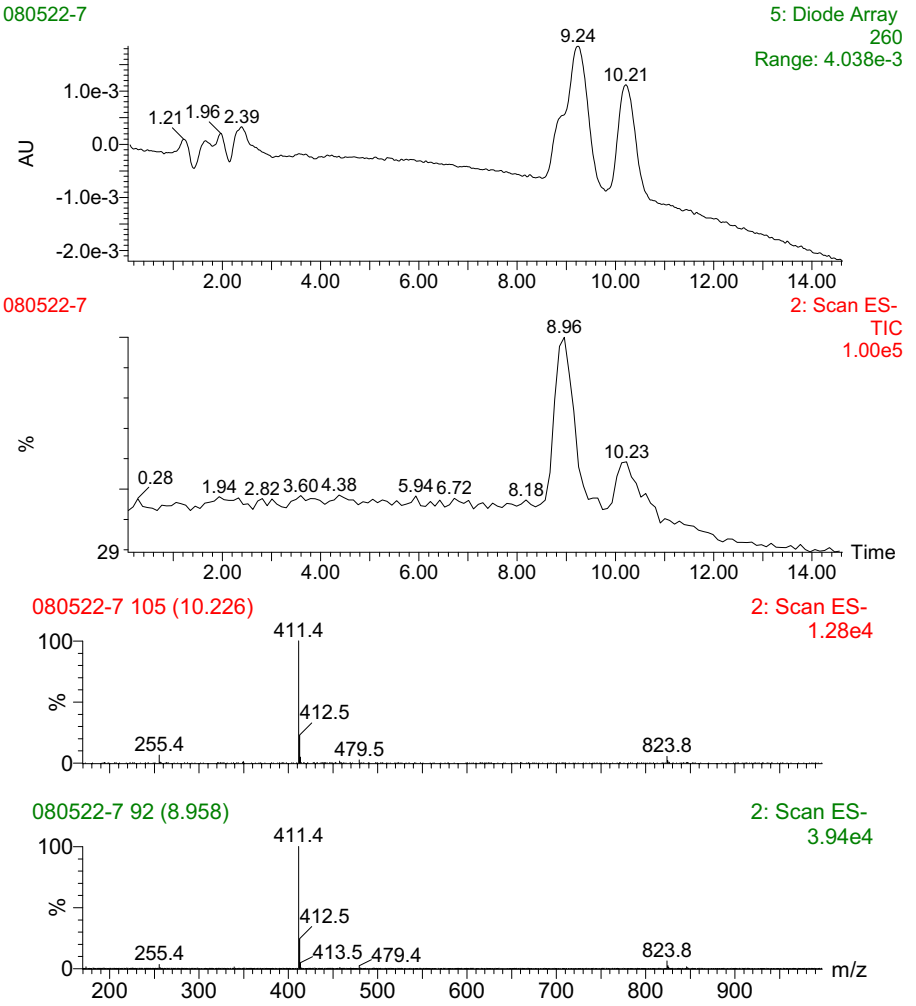


Fig. 3 The HPLC–MS of purified MPSA

Table 1 The influence of MPSA on mice's average weights.

Number of days	Blank control (g)	Treated group (g)
0	23.6	23.6
1	24.6	24.2
2	25.5	24.8
3	27.5	26.5
4	28.0	27.0
5	28.5	27.4
6	28.5	27.4
7	29.0	28.2

angular vein and placed in 10-ml plastic centrifuge tubes which had a decoagulant (1/10 volume of 0.109 mol l^{-1} sodium citrate solution). The tubes were inverted gently a few times to mix well and then centrifuged at 3,000 rpm for 15 min. The supernatants were collected and preserved at −20°C. Then the items were determined according to the kits.

The data were statistically analyzed with SPSS V11.0 and expressed as $\overline{x} \pm s$ and the significance of the difference was determined by single-factor analysis of variance.

Monopalmityloxy Shikimic Acid Efficacy Test: Arteriovenous Loop Thrombosis

After an adaptation period for 3 days, 20 normal SD rats, half males and half females, were randomly divided into three groups: four for control, six for SA treatment group, and ten for MPSA treatment group. The dosage was 0.724 mmol $kg^{-1}\cdot day^{-1}$. The MPSA was dispersed in water with the aid of Tween 10 (0.05 ml/10 ml). The rats in the treatment groups were orally administered continuously for 3 days, while the control group and SA group received the same volume of physiological saline and SA solution (0.575 mmol $kg^{-1}\cdot day^{-1}$), respectively. One hour after the last administration, they were anesthetized with 8% chloral hydrate solution (350 mg kg^{-1}) via i.p. and then supine position fixed. The right carotid and left external jugular were isolated. A pre-weighted, with a length of about 8 cm, seventh surgery thread was put into a polyethylene pipe with the length of 10 cm, which is full of physiological saline, both ends of which were linked with insertion tubes full of heparin (the length was about 3 cm). One of the insertion tubes was inserted in the jugular and another one was inserted in the carotid. The folder of the artery was opened, letting the blood flow through the circuit loop *in vitro* for 15 min. Flow was interrupted after 15 min. Then the thrombus was taken out and weighed quickly. The wet weight of thrombus (W_t) is the difference between the weight weighted here and the weight of thread.

$$\text{Inhibition rate of thrombosis} = \frac{W_{t,\text{blank}} - W_{t,\text{treatment}}}{W_{t,\text{blank}}}$$

The data were statistically analyzed by the same method and software mentioned above.

Table 2 Acute toxic experiment result.

Groups	*n*	Dose (mg kg^{-1})	Weight increase during 7 days (g)	Weight growth rate (%)	Number of deaths
Blank	10	–	5.4	22.9	0
Test	10	1,200	4.6	19.5	0

Table 3 The influence of MPSA on the TT of rats.

Groups	*n*	Dosage (mg kg^{-1} day^{-1})	TT (s)
Control	3	–	16.09±1.46
Aspirin	3	300	20.25±3.44*
MPSA	3	100	19.59±0.801*

Analysis of variance, $P<0.05$; compared with control *$P<0.05$

Results

Enzymatic Synthesis, Purification, and Structure Identification of Monopalmityloxy Shikimic Acid

The HPLC–MS analysis results show us that there is a new compound formed and the new compound formed in this system is MPSA. There are mainly three peaks, 1.63, 21.75, and 22.87 min, in the HPLC of reaction mixture of shikimic acid and palmitic acid with Novozym 435 and the MS spectrum of such peaks corresponds to SA (M-1=173), MPSA (M-1=411), and palmitic acid (M-1=255). The peak at 21.75 min did not occur for the mixture SA and palmitic acids without Novozym 435.

The TLC analysis results (Fig. 2) suggest that the suitable fatty acid substrate range, for the formation of such alkanoloxy derivatives of SA, is quite wide, at least from valeric acid to stearic acid; besides the SA spot, there is another yellow spot which has the Rf between SA and fatty acid. Such spot did not occur for the mixture SA and alkanoic acids without Novozym 435.

After purification, 28.63 g of purified product has been collected. The overall conversion rate was over 70%. The HPLC–MS of purified MPSA are shown in Fig. 3.

Single Dose Acute Oral Toxicity Test of Monopalmityloxy Shikimic Acid

The mice's activities have been observed to reduce during the first hour after oral administration, and then gradually restore to normal level within 4 h. The food intake of the mice in the treatment group has been observed to increase gradually during the period of observation (7 days) with the normal appetite, spirit, and color pattern. The continuous increase in the average weights of the mice in the treatment group (see Table 1) has also been observed. Neither death during the period of observation (7 days) (see Table 2) nor pathological change or toxication in the heart, liver, spleen, lung, and kidney of the tested mice during the anatomization on the seventh day has been observed. Such results show that monopalmityloxy shikimic acids have very little toxicity (the largest safe dose is not less than 1,200 mg kg^{-1}).

Table 4 The influence of MPSA on the PT, APTT of rats.

Groups	*n*	Dosage (mg kg^{-1} day^{-1})	PT (s)	Lengthen (%)	APTT (s)	Lengthen (%)
Control	3	–	14.89±1.52	–	56.44±1.53	–
Aspirin	3	300	21.44±4.87*	43.99	71.31±3.14**	26.35
MPSA	3	100	20.00±2.33*	34.32	71.18±3.14**	26.12

Analysis of variance, $P<0.05$; compared with control *$P<0.05$, **$P<0.01$

Table 5 Thrombus restrain percent.

Groups	*n*	Dosage (mmol kg^{-1} day^{-1})	$\frac{\text{The wet weight of thrombus}}{\text{body weight}} \times 100$	Inhibition of thrombosis (%)
Control	4	–	11.914±0.241	–
SA	6	0.575	10.421±0.758*	12.5
MPSA	10	0.724	10.372±0.801*	12.9

Analysis of variance, $P<0.05$; compared with control *$P<0.01$

Determination of Thrombin Time, Prothrombin Time, and Activated Partial Thromboplastin Time

Compared with control group, the differences of the treatment group and aspirin group of rats are significant ($P<0.05$) for thrombin time. The result shows that MPSA can significantly prolong the thrombin time. The result is shown in Table 3.

Compared with control group, the differences of the treatment group and aspirin group of rats are significant ($P<0.05$) for prothrombin time and very significant ($P<0.01$) for activated partial thromboplastin time. The results show that MPSA can inhibit the generation of thrombin through intrinsic pathway and extrinsic pathway. And the inhibitory action of intrinsic pathway is stronger than that of extrinsic pathway. The result is shown in Table 4.

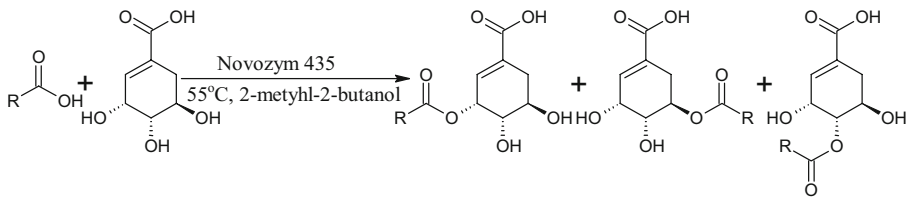
There is no significant difference between the treatment group and aspirin group.

Monopalmityloxy Shikimic Acid Efficacy Test: Arteriovenous Loop Thrombosis

Compared with control group, the differences of the treatment group and SA group of rats are very significant ($P<0.01$) for the ratio of wet weight of thrombus to body weight. The result shows that MPSA 0.724 mmol kg^{-1} day^{-1} via oral administration for 3 days, just like SA, could inhibit thrombosis and elevated the blood coagulation time of rats. The inhibition rate of MPSA (12.9%) is a little bit higher than that of SA (12.5%), but unfortunately, such difference is insignificant ($P>0.05$). The result is shown in Table 5.

Discussion

Shikimic acid is a natural bioactive product with anti-thrombosis activity, but the poor bioavailability and BBB permeability limited its general therapeutic utilization. Through monoacylation, a long fatty acid residue can be bound to offer it a desirable modified polarity. And such series of compounds with different lipophilicity (log P) is desirable to optimize the BBB permeability of liposoluble derivatives of SA. According to the



Scheme 1 The synthesis of MPSA catalyzed by Novozym 435 in 2-methyl-2-butanol

calculation result, the log P (calculated by ChemSketch) of monoacylated SA will increase gradually from −0.85 of monoacetyl SA, through 0.74 of monopentanoyloxy SA, to 7.65 of monostearyl SA with the increase of the chain length of alkanoxyl group.

In this paper, MPSA, chosen as the representative of such series of monoacylated SA with the suitable calculated log P (6.59±0.39) value, neither too low nor to high, has been synthesized in one-step esterification directly from SA and palmitic acids by the reaction shown in Scheme 1. It delivers a new pathway for us to synthesize the series monoalkanoloxyl SA and investigate the relationship between the chain length of fatty acid, the corresponding log P of MASA, and BBB permeability.

Checked by HPLC–MS (Fig. 3), there are two or three products that have been formed, but they all belong to the MPSA derivatives (with the same molecular weight). No diacylated one has been detected. The possible structures of such products are 3-, 4-, or 5-substituted MPSA. In order to characterize and utilize the products as active pharmaceutical intermediates, and to characterize the enzyme for this reaction system, further separation is needed to get the pure single product. The kinetics of this synthesized reaction also needs to be characterized.

Such MPSA has been confirmed to possess the anticoagulation activity *in vivo* via oral administration, with very little toxicity via oral administration (the largest safe dose is not less than 1,200 mg kg^{-1}). Unfortunately, the results are not as good as expected. It could be due to the poor solubility of MPSA in water. In order to check its anticoagulation activity, especially the BBB permeability, the acute toxicity and anticoagulation effects via i.v. need to be evaluated.

Furthermore, as a drug candidate with excellent BBB permeability, the neurotoxicity of MPSA must also be evaluated.

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