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Design and Synthesis of Y-27632 Liver Targeted Prodrug and Its in Vivo Activity Test

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Abstract

Hepatic fibrosis is a complex pathophysiological process, and the key link is the activation of hepatic stellate cells (HSC). Rho/Rock signal pathway is involved in the activation of HSC, and ROCK inhibitor Y-27632 can down-regulate the expression of Rho/Rock, thus relieving the symptoms of liver fibrosis. The function of Rho protein family is complex in human body. If Y-27623 is directly used to inhibit its expression, the drug concentration in liver and the curative effect is low. The structure of Y-27632 was modified by ProTide technology to improve its lack of liver targeting, effectively enrich in the liver, improve its curative effect and reduce its side effects. A series of compounds were synthesized from phenyl dichlorophosphate, diphenyl phosphate, amino acid ester hydrochloride and Y-27632. Their structures were characterized by 1H-NMR, 13C-NMR and HR-MS (ESI).

Keywords

Hepatic fibrosis, hepatic stellate cells, Rock inhibitor, Y-27632, ProTide.

1. INTRODUCTION

Liver disease is the serious problem of public health in China. It is estimated that there are about 400 million patients with various liver diseases in China, among which 86 million are infected persons with chronic hepatitis B virus (HBV), accounting for 23.5%; 10 million infected persons with chronic hepatitis C virus (HCV), accounting for 2.5%; 60 million infected persons with alcoholic liver disease, accounting for 15.0%; 200 million infected persons with non-alcoholic fatty liver disease, accounting for 50.0%; 37 million infected persons with other liver diseases, accounting for 3.9%. Stimulated by chronic inflammation, these patients all have mild or severe symptom of hepatic fibrosis, which will deteriorate into cirrhosis, and even liver cancer with the long-term development. Now, the treatment for anti-hepatic fibrosis is divided into two categories, one is the treatment of etiology, which is mainly to remove the pathogenic factors, such as the treatment for anti-hepatitis B and hepatitis C virus, the treatment for anti-schistosomiasis and alcohol withdrawal; the other is the treatment of hepatic fibrosis, which can inhibit the activation of hepatic stellate cells by means of promoting matrix degradation and inhibiting inflammation or lipid peroxidation. [1], [2], [3], [4] Removing the etiology and preventing liver damage can be only achieved by means of the treatment for the primary disease, but by which reversing the hepatic fibrosis cannot be achieved effectively. Therefore, the further research on the mechanism of hepatic fibrosis is needed to reverse hepatic fibrosis in allusion to the treatment of hepatic fibrosis itself.

Hepatic fibrosis refers to the activation of hepatocytes, macrophages (Kuffer cells), platelets and other cells in the body stimulated by the pathogenic factors, which releases a variety of

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cytokines and activates HSC to increase its fecundity and contractility and release pro-inflammatory factor and pro-fibrotic factor by means of the autocrine process, which increase the mobility of ECM, increase the synthesis rate and decrease the degradation rate, ECM is deposited in large quantities, forming scar tissue to replace the original hepatic tissue[5]. The activation of hepatic stellate cells plays the critical role in the development of hepatic fibrosis, and the excellent guarantee for the treatment of hepatic fibrosis can be provided by means of the further research on the activation mechanism of hepatic stellate cells. According to the experiment Aihara [6], [7] and others, the Rho/Rock signaling pathway mediates the release of peripheral T lymphocytes, which indicates that the Rho/Rock signaling pathway may be involved in the formation of hepatic fibrosis by regulating inflammatory cell infiltration and secretion of inflammatory mediators. The Rho/Rock signaling pathway is also involved in the proliferation and apoptosis of HSC, in the experiment of vitro HSC, the Rho/Rock signaling pathway is involved in mediating actin cytoskeletal recombination of apoptotic cells and macrophages during the entire process of the apoptosis [8], [9]. In addition, the Rho/Rock signaling pathway is involved in the expression of genes related to hepatic fibrosis, such as transforming growth factor TGF-β1 and exchanger (NCX) Na+/Ca2+, etc [10], [11]. Inhibiting the activation and proliferation of hepatic stellate cells is one of the main methods to reverse hepatic fibrosis. The expression of Rho/Rock signaling pathway can be down-regulated by means of Rock inhibitor Y-2763, thereby the activation of hepatic stellate cells can be inhibited. It's found by Iwamoto [12] and others that the expression and deposition of I collagen gene can be reduced by Y - 27632, which indicates that the expression of Rho/Rock signaling pathway can be down-regulated by Y-237632 in various ways for inhibiting the activation of hepatic stellate cells. However, THE Rho/Rock signaling pathway is widely distributed in the human body, involving multiple organs and tissues such as liver, lung and kidney, but Y-27632 lacks hepatic targeting, if y-27632 is directly applied to treat hepatic fibrosis with low intrahepatic drug concentration, which is difficult to achieve predetermined efficacy, in addition, there may be a large number of adverse effects that are difficult to estimate. Therefore, the hepatic targeting system is proposed in response to the proper time and conditions.

Liver targeted drug delivery system is a drug delivery system that the pharmacophore is concentrated in the liver effectively to reduce the damage to other organs in the body, which is mainly divided into 4 categories: active liver targeted system, passive liver targeted system, physicochemical targeted system and prodrug delivery system. The active liver targeted system is the system that delivers pharmacophore directly to the liver with the appropriate modifications, the main mechanisms include receptor-ligand binding or antigen-antibody binding, including the liver targeted drugs mediated by non-saliva acid glycoprotein receptor, liver targeted drugs mediated by bile acid carrier, liver targeted drugs mediated by lipoprotein and liver targeted drugs mediated by immune; the passive hepatic targeted system selects the appropriate carrier to transform pharmacophore into the structure that can be engulfed by macrophages, delivered to the liver by the physiological response, including particles, liposomes and liposome particles; the physicochemical liver targeted preparations are designed to take advantage of the physicochemical properties of the liver with the targeted release of drugs in the liver, which include magnetic carrier preparations and targeted drug therapy for arterial embolization; the liver targeted prodrug delivery system mainly is to transform the pharmacophore into the structure with less pharmacological activity, pharmacophore with pharmacological effects are released by the degradation of specific enzyme after entering hepatocytes to effectively increase the concentration of female drug in liver and reduce the toxic and side effects on other organs. ProTide prodrug technology has attracted wide attention due to its excellent hepatic targeting, good water solubility and membrane permeability, excellent curative effect and low toxicity, etc. Its development can be mainly divided into six stages, including alkyl and halogenated alkyl phosphates, alkoxy and halogenated alkoxy phosphates,

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diamidophosphate, lactate derived systems, diaryl phosphates and aryloxy phosphoramidate [13]. The core of the technology is to splice the original pharmacophore into the phosphate ester with liver targeted property[14], the core of the technology is to splice the original pharmacophore into the phosphate ester with liver targeted properties, the polarity of phosphorus center can be reduced to improve its membrane permeability by means of introducing aromatic substituents and amino acid esters with strong electrical absorption [15], in this way, the female drug can be enriched in liver to solve the problem of lack of hepatic targeting. Several critical drugs have been developed based on this technology, such as Tenefovir Alafenamide (TAF) and Sofosbuvir. There are three main synthetic methods for ProTide, (1) Pharmacophore is monosubstituted with diaryl phosphonate and then aminated and oxidezed with amino acid esters [16]; (2) The pharmacophore is directly coupled to phosphoryl chloride [17]; (3) The medicated aryl phosphate is condensed with amino acids[18]; In addition, ProTide can be prepared by corresponding amino acids, three specific methods are listed as follows: (1) The corresponding phosphoric acid is taken as the substrate, followed by double chlorination, and then phenol and amino acids were added in order with the rate 1:1; (2) the corresponding phosphoric acid is taken as the substrate, catalyzed by dicyclohexyl carbon diimide, condensed with phenol to form chlorophosphate, and then coupled with amino acids; (3) the corresponding diphenyl phosphate is taken as the substrate, a molecule of phenyl ester is first removed and then coupled with amino acids. The ProTide prodrug technology is adopted in this research group, Y-27632 is directly coupled with phosphoacyl chloride and transformed into the structure of phosphoamide ester, which can improve the lack of targeting of Y-27632 and improve its efficacy in the treatment of anti-hepatic fibrosis. A series of chemical structures such as 5a~3d and 8a~8b have been compounded, of which structure is characterized by 1H-NMR, 13C-NMR and HR-MS(ESI).

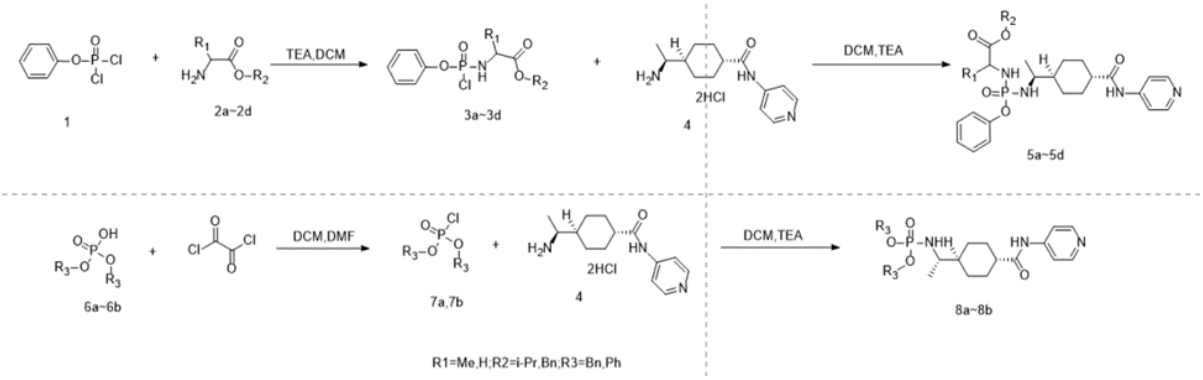

Figure 1. Synthetic route

2. EXPERIMENT

2.1. Instruments and reagents

WFH-203B Triple-Purpose Ultraviolet Analyzer, BRUKER AVANCE IIIHD400M Nuclear Magnetic Resonance Spectrometer and Thermo Fisher Scientific LCQ Mass Spectrometer;

The reagents applied are all analytically pure, all solvents are strictly dehydrated and dried.

2.2. Compounding

(1) Dredging method of compounding for 5a-5d

20mL of anhydrous dichloromethane, 149.4 L (1mmol) phenyl dichlorophosphate and 168mg (1mmol) L-alanine isopropyl hydrochloride are added with stirring under the protection of Nitrogen, 10mL of dichloromethane with 277.3 L (2mmol) triethylamine slowly is added at -40°C, stirring at -40°C for 1h, then moved to room temperature for 1h, 192mg

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(0.6mmol) Y-27632 dihydrochloride is added, 20mL dichloromethane solution with 332.6 L (12.4mmol) triethylamine is slowly added, overnight at room temperature (reaction time is about 16h), spin-dry reaction liquid, add 20mL water and 10mL ethyl acetate to extract for four times and combine the organic phase, dried by anhydrous sodium sulfate, silica gel column chromatography is applied after the enrichment. [Eluent: V (dichloromethane) /V (methanol) =10/1] 191.30mg white solid can be obtained after purification.]

(2) Compounding of 7a~7b

Add 10mL anhydrous dichloromethane and 6a ~6b(5mmol) into a 50m round bottom flask, oxaloyl chloride (1057.7μ,12.5mmol,C3) and DMF (catalytic amount) are added at 0°C (ice bath), stir at room temperature for 1 hour. When the reaction is over, the reaction liquid is spun dry, silica gel column chromatography [Eluent: V (dichloromethane) /V (methanol) =10/1] is applied after purification, and 7a~7b can be obtained.

(3) Dredging method of compounding for 8a~8b

10mL of anhydrous dichloromethane, 7a~7b (1.14mmol), Y27632 dihydrochloride (182mg,0.56mmol) and triethylamine (233 L, 1.68mmol) are added to a 50mL round bottom flask, stir at room temperature for 1 hour, TLC is adopted to monitor the reaction without any change, and the reaction liquid is spun dry, add 10mL water and 5mL ethyl acetate to extract for 3 times and combine the organic phase, dried by anhydrous sodium sulfate, after filtration and concentration, silica gel column chromatography [Eluent: V (dichloromethane) /V (methanol) =10/1] is applied after purification, 8a~8b can be obtained.

N-[P-4-[(1R)-1- Amino ethyl]-N-(pyridine-4-radical) cyclohexane -1- Formyl amino-P-phenyl]-L- Alanine isopropyl ester (5a), yield is 28.5%, 1H NMR (400 MHz, CDCl3) δ 9.44 (d, J = 38.5 Hz, 1H, -NH), 8.38 (dd, J = 5.3, 4.0 Hz, 2H, -2PyH), 7.56 (dd, J = 7.5, 2.8 Hz, 2H, -2PyH), 7.30 (d, J = 7.9 Hz, 1H, -ArH), 7.18 (ddd, J = 33.3, 20.4, 12.0 Hz, 4H, -4ArH), 5.01 (m, 1H, -CH), 3.97 (m, 1H, -CH), 3.67 (m, 2H, -2NH), 3.15 (d, J = 6.6 Hz, 1H, -CH), 2.81 (ddd, J = 21.0, 18.3, 10.5 Hz, 1H, -CH), 2.30 (m, 1H, -CH), 1.95 (d, J = 12.8 Hz, 3H,-3CH), 1.77 (t, J = 13.4 Hz, 1H, -CH), 1.52 (m, 2H, -2CH), 1.37 (dt, J = 12.2, 7.3 Hz, 3H, -CH3), 1.23 (m, 6H, -2CH3), 1.14 (m, 3H, -CH3), 0.97 (m, 2H, -2CH). 13C NMR (101 MHz, CDCl3) δ 175.92, 175.84, 173.91, 173.85, 173.79, 173.72, 151.22, 151.15, 150.10, 146.20, 129.63, 124.62, 124.52, 120.35, 120.31, 120.21, 120.16, 120.13, 113.75, 70.50, 69.31, 69.26, 52.42, 52.25, 52.15, 50.41, 50.11, 45.85, 45.75, 43.81, 43.74, 43.68, 29.68, 29.35, 29.15, 28.99, 28.84, 28.45, 28.32, 28.19, 27.89, 22.68, 21.75, 21.73, 21.64, 21.17, 21.11, 20.86, 14.12, 8.65. 31P NMR (162 MHz, CDCl3) δ 8.84, 8.64, 8.50, 8.29.ESI-MS(m/z):calcd for C26H37N4O5P [M+H]+ 517.25, found 517.79.

N-[P-4-[(1R)-1- Amino ethyl]-N-(pyridine-4-radical) cyclohexane -1- Formyl amino-P-phenyl]-L- Benzyl glycine ester(5b), yield is 27.6%, 1H NMR (400 MHz, CDCl3) δ 9.72 (d, J = 22.6 Hz, 1H, -NH), 8.37 (s, 2H,-2PyH), 7.73 (s, 2H, -2PyH), 7.32 (m, 6H, -6ArH), 7.16 (m, 4H, -4ArH), 5.15 (m, 2H, -CH2), 3.78(m, 2H, -2NH), 3.66 (s, 2H,-CH2), 2.30 (s, 1H, -CH), 1.93 (d, J = 11.1 Hz, 3H, -3CH), 1.72 (s, 1H, -CH), 1.47 (m, 2H, -2CH), 1.35 (dd, J = 9.0, 5.5 Hz, 2H, -CH2), 1.09 (dd, J = 10.3, 6.5 Hz, 3H, -CH3), 0.91 (m, 2H, -2CH). 13C NMR (101 MHz, CDCl3) δ 176.19, 171.20, 151.02, 148.95, 1350.04, 129.70, 128.69, 128.65, 128.44, 128.43, 124.67, 120.37, 120.32, 120.26, 120.21, 114.25, 67.32, 45.93, 45.82, 43.52, 43.13, 31.44, 29.70, 29.36, 28.89, 28.19, 27.59, 20.76, 14.13, 8.65. 31P NMR (162 MHz, CDCl3) δ 9.80, 9.64.ESI-MS(m/z):calcd for C29H35N4O5P [M+H]+ 551.23, found 551.22.

N-[P-4-[(1R)-1- Amino ethyl]-N-(pyridine-4-radical) cyclohexane -1- Formyl amino-P-phenyl]-L- Glycine isopropyl ester(5c), yield is 23.2%, 1H NMR (400 MHz, CDCl3) δ 9.77 (d, J = 24.2 Hz, 1H, -NH), 8.35 (s, 2H, -2PyH), 7.61 (d, J = 3.7 Hz, 2H, -2PyH), 7.20 (ddd, J = 29.6, 14.5, 7.5 Hz, 5H, -5ArH), 5.06 (m, 1H, -CH), 3.78(s, 1H, -CH), 3.72 (d, J = 10.5 Hz, 2H, -2NH), 3.17 (d, J = 8.8 Hz, 1H, -CH), 2.28 (s, 1H, -CH), 1.93 (d, J = 10.3 Hz, 3H, -3CH), 1.76 (d, J = 9.3 Hz, 1H, -CH), 1.48 (t, J = 19.0 Hz, 2H, -2CH), 1.23 (d, J = 6.2 Hz, 6H, -2CH3), 1.13 (d, J = 10.8 Hz, 3H, -CH3), 0.94

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(ddd, J = 68.7, 34.4, 22.4 Hz, 2H, -2CH). 13C NMR (101 MHz, CDCl3) δ 176.23, 176.16, 171.12, 170.87, 170.80, 151.11, 151.04, 149.23, 147.03, 129.68, 124.71, 124.67, 120.36, 120.31, 120.23, 120.18, 113.91, 69.51, 52.34, 52.17, 45.77, 43.58, 43.25, 43.19, 29.69, 29.05, 28.94, 28.83,28.57, 28.34, 27.72, 21.75, 20.88, 14.14. 31P NMR (162 MHz, CDCl3) δ10.07, 9.91.ESI-MS(m/z): calcd for C25H35N4O5P [M+H] + 503.23, found 503.14.

N-[P-4-[(1R)-1- Amino ethyl]-N-(pyridine-4-radical) cyclohexane -1- Formyl amino-P-phenyl]-L- Benzyl alanine(5d), yield is 49.6%, 1H NMR (400 MHz, CDCl3) δ 9.89 (d, J=13.4Hz, 1H, -NH), 8.35 (s, 2H, -2PyH), 7.62 (s, 2H, -2PyH), 7.25 (m, 10H, -10ArH), 5.12 (m, 2H, -CH2), 4.06(m, 1H, -CH), 3.76 (m, 2H, -2NH), 3.06(m, 1H, -CH), 2.98(m, 1H, -CH), 2.26 (s, 1H, -CH), 1.89 (s, 3H, -3CH), 1.71 (s, 1H, -CH), 1.48 (d, J = 10.8 Hz, 2H, -2CH), 1.38 (m, 3H, -CH3), 1.07 (m, 3H, -CH3), 0.91(m, 2H, -2CH). 13C NMR (101 MHz, CDCl3) δ 176.21, 174.14, 174.09, 151.20, 151.14, 151.09, 149.63, 147.24, 135.14, 129.66, 128.70, 128.68, 128.68, 128.59, 128.41, 128.25, 128.19, 124.70, 124.61, 120.38, 120.34, 120.23, 120.18, 120.11, 114.07, 67.27, 53.50, 52.41, 52.28, 52.18, 50.39, 50.15, 45.72, 45.66, 43.60, 29.06, 28.92, 28.80, 28.43, 28.30, 28.08, 27.93, 21.03, 20.98, 20.86. 31P NMR (162 MHz, CDCl3) δ 8.82, 8.62, 8.49, 8.32.ESI-MS(m/z): calcd for C30H37N4O5P [M+H]+ 564.25, found 565.21.

4-[(1R)-1- Amino ethyl]-N-(pyridine-4-radical) cyclohexane -1- Dibenzyl formyl aminophosphate) (8a), yield is 58.2%, 1H NMR (400 MHz, CDCl3) δ 9.03 (s, 1H, -NH), 8.42 (d, J = 5.7 Hz, 2H, -2PyH), 7.58 (t, J = 11.2 Hz, 2H, -2PyH), 7.35 (m, 10H, -10ArH), 5.05 (m, 4H, -2CH2), 3.09 (m, 1H, -NH), 2.62 (dd, J = 12.6, 8.2 Hz, 1H, -CH), 2.24 (t, J = 12.0 Hz, 1H, -CH), 1.96 (t, J = 14.9 Hz, 3H, -3CH), 1.75 (d, J = 12.6 Hz, 1H, -CH), 1.54 (m, 2H, -2CH), 1.11 (d, J = 6.6 Hz, 3H, -CH3),0.89 (m, 2H, -2CH). 13C NMR (101 MHz, CDCl3) δ 175.54, 175.42, 149.98, 149.75, 146.17, 145.85, 136.58, 136.30, 136.30, 136.27, 136.23, 136.20, 128.62, 128.42, 127.56, 113.77,68.21, 68.16, 68.12, 52.39, 46.00, 43.57, 43.51, 29.70, 29.02, 28.94, 28.32, 27.69, 20.67, 20.65. 31P NMR (162 MHz, CDCl3) δ8.69.ESI-MS(m/z):calcd for C28H34N3O4P [M+H]+ 508.23, found 508.18.

4-[(1R)-1- Amino ethyl]-N-(pyridine-4-radical) cyclohexane -1- Diphenyl formyl phosphoramidic acid(8b), yield is 55.3%, 1H NMR (400 MHz, CDCl3) δ 9.04 (d, J = 4.0 Hz, 1H, -NH), 8.35 (m, 2H, -2PyH), 7.45 (dd, J = 4.9, 1.6 Hz, 2H, -2PyH), 7.32 (m, 4H, -4ArH), 7.20 (m, 6H, -6ArH), 3.28 (m, 1H, -NH), 3.17 (td, J = 10.9, 6.5 Hz, 1H, -CH), 2.19 (td, J = 11.9,2.7 Hz, 1H, -CH), 1.94 (t, J = 12.3 Hz, 3H, -3CH), 1.78 (d, J = 12.1 Hz, 1H, -CH), 1.50 (m, 2H, -2CH), 1.15 (d, J = 6.5 Hz, 3H, -CH3), 0.96(ddd, J = 15.5, 11.0, 3.7 Hz, 2H, -2CH). 13C NMR (101 MHz, CDCl3) δ 175.54, 150.87, 150.82, 150.80, 150.75, 150.27, 145.84, 129.79, 129.74, 125.18, 125.16, 120.20, 120.16, 113.67, 53.20, 45.80, 43.73, 43.66, 29.70, 28.98, 28.91, 28.52, 27.85, 20.79, 20.76. 31P NMR (162 MHz, CDCl3) δ -1.08, -1.12.ESI-MS(m/z): calcd for C26H30N3O4P [M+H]+ 480.20, found 480.20.

3. RESULTS AND DISCUSSION

3.1. Compounding

In consideration of the low yield of the reaction and the high price of the reaction material Y-27632, the reaction conditions have been optimized to improve the utilization of Y-27632.

It can be seen from Table 1 that No.1~4 is the optimization of reaction time, the yield is the highest at 16h under the same conditions; No.3 and No.5~7 are optimization of reaction temperature conditions, the yield is the highest at -40°C under the same conditions; No.3 and No.8~10 are the optimization of reaction concentration conditions, the yield of Y-27632 is the highest at 50mmol/ L under the same conditions. To sum up, the yield is the highest when the reaction time is 16h, the reaction temperature is -40°C and the reaction concentration is 50mmol/ L.

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Table 1. Optimization of compounding conditions for 5a

No	Boric acid /eq	Time/h	Temperature/°C	Concentration /(mmol/μL)	yield/%
1	\	8	-40	50	13
2	\	12	-40	50	15
3	\	16	-40	50	28
4	\	24	-40	50	12
5	\	16	-20	50	0
6	\	16	-60	50	18
7	\	16	-80	50	15
8	\	16	-40	12.5	8
9	\	16	-40	25.0	22
10	\	16	-40	100.0	17
11	0.05	16	-40	50	15
12	0.05	16	-40	50	20

Note: The reaction temperature here is the first reaction temperature, and the reaction time starts from the addition of Y-27632 dihydrochloride.

In addition, add the excess alkali is added in the neutralization reaction of weak acid boric acid to investigate whether the excess alkali has an effect on the reaction yield. Firstly, in the reaction stage, after adding the raw material Y-27632 dihydrochloride, boric acid of 0.05eq is added, the system is post-treated after stirring at room temperature for 16h, and the reaction yield is 15% (in Table No.11). Then the order of adding boric acid is adjusted, according to the original experimental conditions, the boric acid washing reaction system of 0.05eq is added in the post-treatment stage, the reaction yield is 20% (in Table No.12). It's showed according to the experimental results that neither of the two schemes can effectively improve the reaction yield, excluding the influence of excessive alkali on the reaction yield.

In addition, after the reaction is over, a tert-butyloxycarbonyl is introducd by means of di-tert-butyl dicarbonate ester to the primary amine of Y-27632, which did not participate in the reaction, and the protecting group is removed in the HCl/MeOH system to recycle Y-27632 further. In this process, it's found that the utilization ratio of y-27632 dihydrochloride prepared by one-pot method is not high, so the process is conducted that phenoxy chlorophosphamide ester is purified in the first step, then Y-27632 dihydrochloride is added for the next reaction, the yield based on Y-27632 is 64.54%. Although the total yield of the reaction is not much different from the previous one, fortunately, the yield was significantly increased compared with Y-27632 with great cost savings.

4. CONCLUSION

The treatment of hepatic fibrosis is limited by the complexity of liver function, and the progress has been slow. More and more attention has been paid to liver targeted therapy, liver targeted therapy is characterized by less damage to other organs in human body and higher efficacy compared with traditional therapy. Rock inhibitor Y-27632 is transformed into ProTide in this article to improve its lack of hepatic targeting, and a series of compounds have been compounded, the reaction conditions have been optimized based on the original experiment, which have improved utilization rate of Y-27632 with great cost savings and provided some reference value for the subsequent research and development of new liver targeted prodrug.

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