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Synthesis and Biological Assessment of 3,7-Dihydroxytropolones

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Abstract

3,7-Dihydroxytropolones (3,7-dHTs) are highly oxygenated troponoids that have been identified as lead compounds for several human diseases. To date, structure-function studies on these molecules have been limited due to a scarcity of synthetic methods for their preparation. New synthetic strategies towards structurally novel 3,7-dHTs would be valuable in further studying their therapeutic potential. Here we describe the successful adaptation of a [5+2] oxidopyrylium cycloaddition/ring-opening for 3,7-dHT synthesis, which we apply in the synthesis of a plausible biosynthetic intermediate to the natural products puberulic and puberulonic acid. We have also

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Conflict of Interest

RM, LM, and JT are coinventors on a patent application that covers anti-HBV and anti-HSV activity of hydroxytropolones.

Author Contributions

DH, AB, DS, and RM conducted the work associated with the synthesis. TM, JW, JB, SL obtained data associated with HIV. EL and JT obtained data associated with HBV. LM, BP, HD, SH, and SM obtained data associated with HSV. TM, HZ, LT, and SL obtained data associated with pUL15C. RP and EG are responsible for *in silico* modelling. DH and RM wrote manuscript with primary assistance and feedback from SL, JB, LM, JT, LT and EG.

tested these new compounds in several biological assays related to human immunodeficiency virus (HIV), hepatitis B virus (HBV) and herpes simplex virus (HSV) in order to gain insight into structure-functional analysis related to antiviral troponoid development.

Graphical abstract

A new 3,7-dihydroxytropolone synthetic strategy provides access to a proposed natural product precursor and potent antiviral compounds.

Introduction

7-Hydroxytropolones (α HTs) and 3,7-dihydroxytropolones (3,7-dHTs) (Scheme 1A) are troponoids with three and four contiguous oxygen atoms, respectively, and display an extraordinarily broad range of biological activity.¹ While this activity is most often attributed in both cases to their ability to serve as metal-binding fragments for many physiologically relevant dinuclear metalloenzymes (Scheme 1A), stark differences in bioactivity have been observed between them. For example, α HTs appear to be substantially more potent than 3,7-dHTs as inhibitors of HIV ribonuclease H,^{2,3} a promising target for HIV antivirals that remains untargeted clinically, as well as aminoglycoside-2''-O-nucleotidyltransferase,⁴ one of the most common determinants of enzyme-dependent aminoglycoside resistance in *Pseudomonas aeruginosa*. On the other hand, the 3,7-dHT natural product puberulic acid has demonstrated potent antimalarial activity, with selectivity on par with clinical agent artesunate, whereas in similar assays α HTs have substantially lower potency and selectivity.⁵ 3,7-dHT also possesses activity against B16 melanoma cells an order of magnitude greater than α HTs, and increases the lifespan of mice bearing B16 melanoma comparably to mice administered the chemotherapeutic agent mitomycin C.⁶

In order to better understand the nuances of structure-bioactivity differences between these classes of troponoids, efficient synthetic methods are needed to access both classes, and of particular value would be those that also allow comparable substitution.⁷ During the preparation of this manuscript, a galactose-based method previously used by Sunazuka and Omura to synthesize puberulic acid⁸ was further adapted to develop a small library of both 3,7-dHTs and α HTs (Scheme 1B), as well as other related troponoids.⁹ Testing of these molecules for their antimalarial activity revealed that a carboxylic acid was key to providing selectivity against *Plasmodium falciparum* K1 versus MRC-5 cells, and it did so whether on an α HT or a 3,7-dHT, but provided more selectivity with 3,7-dHT. These studies highlight

the value in a divergent, unifying strategy in SAR determination of oxygenated troponoids, and their importance in assessing the inhibitory potential of these oxygenated troponoids.

Our lab has previously reported an extremely efficient oxidopyrylium cycloaddition/ring-opening strategy for α HT synthesis that has to date generated over 50 published α HTs with varying substitution patterns,^{10,11} and has been used to provide SAR and pursue synthetic chemistry-based optimization studies related to various human diseases^{12–18}. We herein report the adaptation of this route to access a series of 3,7-dHTs. We highlight this strategy through the synthesis of a potential biosynthetic precursor to the 3,7-dHT natural products puberulonic and puberulic acid. The molecules were assessed as part of biochemical and biological studies related to HIV, hepatitis B virus (HBV), and herpes simplex virus (HSV)-1 and -2. These studies help provide an understanding of the advantages and disadvantages of 3,7-dHTs and α HTs as chemotypes for drug discovery and development.

Results and Discussion

Synthesis

Our synthesis started with a cycloaddition between oxidopyrylium triflate salt **1a**, which can be made on gram scale in 3 steps from commercially-available kojic acid,¹⁹ and iodopropiolates (Scheme 2A, 2a/b), which are prepared from the corresponding propiolates using silver nitrate and N-iodosuccinimide.^{20,21} Bromopropiolates are also capable of efficient oxidopyrylium cycloaddition and have been used in the synthesis of bromohydroxytropolones,¹⁴ but their volatility and strong lachrymator properties pose technical challenges. The iodobicycles can then be converted into methoxybicycles through a DMAP-catalyzed methanolysis (**3a/b** $\rightarrow$ **4a/b**). These conditions were deemed necessary when we found that Brønsted base-mediated methanolysis led to dimethyl acetals (*e.g.* **9**), while DMAP incorporation was observed as the major product when chloroform was employed as the solvent in an attempt at hydrolysis (**8**, Scheme 2B). Interestingly, subsequent ring-opening attempts on these bicycles (**4a/b** $\rightarrow$ **5a/b**) using sulfonic acids led to formation of oxidopyrylium dimers as the major product, as observed by crude ¹H NMR (*e.g.* **7a**, Scheme 2C). We hypothesize that this may proceed via protonation and elimination of the β -methoxyenoate, which would lead to a rapidly dimerizing oxidopyrylium ylide.²² Fortunately, an alternative procedure using the Lewis acid boron trichloride promoted clean conversion to 3,7-dimethoxytropolones **5a** and **5b**, which were subsequently converted to the 3,7-dHTs **6a** and **6b** using hydrobromic acid in acetic acid at elevated temperatures. Additionally, in the presence of excess water along with extended reaction times, methyl ester **5a** was readily decarboxylated (**6c**, Scheme 2D), which can be attributed to the participation of a β -keto acid tautomer. Thus, while the ester provides a convenient synthetic handle for efficient oxidopyrylium cycloaddition and subsequent iodide for methoxide exchange, it can be readily removed if desired.

With an efficient method for 3,7-dHT synthesis in hand, we sought to test the new strategy in total synthesis. The two most widely studied 3,7-dHTs are puberulonic and puberulic acid, which have been isolated from the fungus *Penicillium puberulum*.²³ These natural products are also close structural homologues of the 6-hydroxytropolones stipitatic and stipitatic acid,²⁴ the focus of recent biosynthetic studies by Cox and coworkers.²⁵ These

studies demonstrated that stipitatic acid is formed from stipitatic acid, which is, in turn, formed from the lactone stipitalide (Scheme 3). While a similar biosynthetic pathway seems plausible for puberulonic and puberulic acid, the 3,7-dHT stipitalide homologue **6d** is unknown. Access to this molecule could be useful in elucidating the biosynthesis of puberulic and puberulonic acid, and our synthetic strategy appeared uniquely well suited for this task. Thus we set out to conduct a total synthesis of **6d**.

We began our synthesis with the chloromethylene-containing oxidopyrylium salt **1b** (Scheme 4). Initial attempts towards bicycle **3c** using our prior conditions ('a', Scheme 4) lead to a significant amount of compound **3b**, which created purification challenges given the similar polarities of **3b** and **3c**. We hypothesized that this product was formed through a hydride transfer from the *N,N*-diisopropylaniline (Scheme 2A),²⁶ and thus, leveraging the readily reversible nature of this class of oxidopyrylium ylides (formed following deprotonation of **1b**),¹⁰ we synthesized and isolated dimer **7b** as the source of the ylide.²⁷ Indeed, this alternative procedure led to the dihalogen **3c** without any noticeable **3b**. While this intermediate offered two electrophilic handles, namely a β -iodoacrylate for methanolysis and a primary alkyl chloride for eventual lactonization, we reasoned that the alkyl chloride would be stable to the methanolysis conditions because of the adjacent tertiary centre. Indeed, methanolysis of **3c** proceeded efficiently while maintaining the chloride's integrity, even though higher temperatures were needed for this transformation than for **3a/b**. Subsequent ring-opening afforded methoxytropolone **5c**, which was then advanced to **6d** through a three-step acetolysis/lactonization/demethylation sequence. During the course of these studies, we also discovered that reverse-phase chromatography of **5c** facilitated on-column lactonization to afford **5e**, providing a more direct synthetic method.

Biology

One of the more active areas of research in our group has been α HT antiviral development, as several viral nucleases have been identified as targets for these molecules.^{2,3,13–18} The most widely studied in these contexts is the ribonuclease H function of HIV reverse transcriptase (HIV RT RNaseH),^{3,13,28–30} a promising candidate for therapeutic development that remains untargeted clinically. α HTs are potent inhibitors of the enzyme with 50% inhibition concentrations (IC₅₀) less than 200 nM, and several synthetic α HTs have been developed with modest antiviral activity in cell-based assays.^{13,28,29} While both 3,7-dHTs and α HTs are known inhibitors of the enzyme, and the collection of literature sources indicate that α HTs are superior inhibitors, head-to-head comparisons of both classes of molecules have not to the best of our knowledge been conducted. We thus tested 3,7-dHTs **6a–6d**, α HT congener **10**, and methoxytropolone congeners **5a** and **11** for their ability to inhibit HIV-1 RT RNaseH. Consistent with literature trends, α HT **10** was the most potent of the compounds tested, with a 4–5 fold increase over the 3,7-dHTs (Figure 1). Methoxytropolones were even less potent, consistent with tropolone activity. Furthermore, while promising cell-based HIV antiviral activity of 3,7-dHT has been described in the literature,³ our synthetic 3,7-dHTs showed no protective effects, possibly due to a combination of weaker anti-RNaseH activity and higher cytotoxicity. Compound **10**, which is known to have modest HIV protective effects,¹³ was tested simultaneously as a positive

control. Thus, early indications suggest that the extra hydroxyl in 3,7-dHTs is unfavourable for HIV therapeutic development targeting RNaseH.

Hepatitis B also has an RNase H function that has become a target for antiviral drug development, and α HTs have emerged as one of the most promising scaffolds.³¹ A 3,7-dHT has never been tested for this activity, and thus we assessed our new compounds' HBV RNase H-specific antiviral activity by monitoring the amount of viral (+)-DNA strand versus (-)-DNA, since inhibiting RNase H activity suppresses production of the former. As expected, α HT **10** showed only minor cytotoxicity against the host cell line, HepDES19, while selectively inhibiting synthesis of the viral (+)-DNA. Selective inhibition of viral (+)-DNA was also observed with 3,7-dHTs **6a** and **6b**, although they were significantly more cytotoxic. It is worth noting the structural similarities of the two selective inhibitors, **6a** and **6b**, to compound **10** and the lack of activity of compound **6c**, which could indicate an important role of the carbonyl appendage. Unfortunately, the EC₅₀ values of the viral suppression of **6a/b** coupled with their CC₅₀ values revealed virtually no therapeutic window, as compared to the 60-fold window of α HT **10**. Thus, while these experiments demonstrate antiviral potential for 3,7-dHTs that may warrant further studies, early indications suggest they are not advantageous over α HTs. On the other hand, the 3,7-dimethoxytropolone **5a** displayed moderate inhibition selectivity for (+)-DNA, had an antiviral EC₅₀ value of 5.5 μ M, and was non-toxic at concentrations of up to 100 μ M. Thus, 3,7-dimethoxytropolone appears to be a viable chemotype for further anti-HBV development.

While 3,7-dHTs had never been tested for anti-HSV activity prior to our studies, α HTs were identified as anti-HSV agents, with the natural product manicol displaying antiviral EC₅₀ values against HSV-1 and -2 of 350 nM and 580 nM, respectively.³² Furthermore, tests against 20 synthetic α HTs synthesized through our oxidopyrylium cycloaddition/ring-opening procedure identified a synthetic α HT with antiviral EC₅₀ values against HSV-1 and -2 of 120 nM and 80 nM, respectively (see **12**, Figure 3).¹⁵ In all cases, cytotoxicity was not observed against Vero cells within the 24 hour experiment. We thus tested our library of tropolones and found that 3,7-dHTs **6a**, **6b**, and **6c** all strongly inhibited replication of HSV-1 at a concentration of 5 μ M, with suppression levels comparable to the therapeutic anti-HSV agent acyclovir (ACV) and exceeding that of cidofovir (CDV) (Figure 2A). α HT **10** only showed moderate replication inhibition at 5 μ M, and no activity at 1 μ M, whereas the activity for **6a–6c** was maintained. Methoxytropolones **5a** and **11** were the least active compounds among the series tested. 3,7-dHT **6d** was inactive, illustrating how changes in the side-chains can impact activity. 3,7-dHTs **6a–6c** inhibited HSV-2 replication with comparable suppression levels and potencies, and this activity was maintained against three other HSV-2 clinical isolates at 5 μ M (Figure 2B). The EC₅₀ values of compounds **6a** and **6c** were comparable to acyclovir for HSV-1, and showed no cytotoxicity versus the Vero cell line at up to 100 μ M for 24 hours (Figure 2C). Furthermore, while ACV was nearly an order of magnitude less potent against HSV-2 than HSV-1, **6a** and **6c** maintained significant potency.

Although the mechanism of action is currently under investigation, experiments with **6a** against the ACV-resistant TK⁻HSV-1 and HSV-2 strains confirmed a different mechanism of

action than ACV (Figure 2B). One hypothesis is that the molecules could engage one or more viral nucleotidyltransferases. For example, the HSV-1 DNA packing terminase pUL15 encodes a C-terminal nuclease activity that can be inhibited by α HTs.^{34,14} Thus, we tested 3,7-dHTs against recombinant pUL15C and found them to be inhibitors of the enzyme, but with less potency than α HT **10** (Figure 2C).

Given the SAR suggesting benefits of the additional oxygen for HSV inhibition potency, a 3,7-dHT analogue of the most potent anti-HSV α HT described to date, compound **12**, was synthesized (**6e**, Scheme 5). Anti-HSV-1 activity was assessed in parallel with 3,7-dHT **6a** and α HTs **12** and **10** (Figure 3B). As expected, compound **10** showed no antiviral activity, even at 5 μ M, whereas some viral suppression was observed for **12**, **6a** and **6e**. At 1 μ M, **6e** and **12** antiviral activity remained at maximum, but activity of **6a** began to drop. At 200 nM, **6a** was inactive, and both **12** and **6e** began to lose some activity. EC₅₀ values of **6e** were ~50 nM against both HSV-1 and -2, which is the most potent anti-HSV activity of a tropolone we have found to date. Unfortunately, moderate cytotoxicity of **6e** was also observed.

While increases in potency against HSV were anticipated through additive effects, surprising effects were observed with pUL15 inhibition. While both a change from an α HT to a 3,7-dHT (**10** $\rightarrow$ **6a**), and from a methyl ester to a biphenyl ketone (**10** $\rightarrow$ **12**) resulted in a decrease in pUL15 potency, changing both provided an increase in potency (**10** $\rightarrow$ **6e**). One hypothesis for this change is that the added hydroxyl permits the biphenyl side chain to adopt a new configuration that would promote new favourable contacts (Figure 4A vs. B). *In silico* modelling was thus carried out by superimposing the active site of crystal structure of pUL15C (PDB id 4IOX)³⁴ to the active site of the crystal structure of HIV RT RNase H bound to manganese cations and β -thujaplicinol (PDB id 3K2P).³⁵ The resulting structure of pUL15C was refined by energy minimization and simulated annealing using the Impact molecular modeling program.³⁶ The receptor grid was generated using the default parameters available in the Schrodinger Suite 2016-3 with one special adjustment where metal constraints were applied to allow metal-ligand interaction at the binding site. Glide docking³⁷ were performed with the ligands α HT **12** and 3,7 dHT **6e**. Several binding poses were obtained for the molecule **6e**, out of which the best binding pose is shown in Fig. 4.

This conformation reveals favorable π -cation interaction between the quaternary ammonium cation of Lys 640 and the phenyl ring π system of the biphenyl group of the ligand. The biphenyl group is thought to be further stabilized by accommodating within a hydrophobic groove formed between Lys 640 and Asn 583 with possible hydrophobic interaction with the Leu 636 present inside the groove. Further stabilization could also be achieved through interactions of the biaryl side chain with Asn 583 through NH- π interactions. These interactions are never observed in *in silico* modelling with **12**. While further studies are needed to understand the specific mechanism of this increase in activity, these analyses highlight the consequences of the additional binding modes allowed by 3,7-dHTs, which might also result in greater likelihood of off-target effects and be a reason for the higher cytotoxicity observed with this class of tropolones.

Conclusions

We have developed an oxidopyrylium cycloaddition/ring-opening approach for the synthesis of 3,7-dHTs. This strategy was applied in the synthesis of a prospective biosynthetic precursor to the natural products pubulonic and puberulic acid. Finally, the new 3,7-dHTs were tested for antiviral activity against HIV, HBV and HSV. Through these studies we found that 3,7-dHTs are weaker inhibitors than α HTs against HIV RNaseH, revealed 3,7-dimethoxytropolone as a promising chemotype for HBV-based antiviral development, and demonstrated that 3,7-dHTs can have greater potency against HSV than analogous α HTs. Furthermore, enzymatic inhibition studies coupled with preliminary *in silico* modelling highlight how additional configurations of 3,7-dHTs may manifest themselves in unexpected potency increases, and could also be responsible for observed cytotoxicity increases

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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B

	HIV-1 RT RNaseH (IC ₅₀ , μM) ^a	HIV -1/ CEM-SS		HBV/ HepDES19	
		antiviral (EC ₅₀ , μM) ^b	cytotoxicity (CC ₅₀ , μM) ^c	antiviral (EC ₅₀ , μM) ^d	cytotoxicity (CC ₅₀ , μM) ^e
6a	1.5 ± 0.02	n.p.	1.4	(**) 6.0 ± 6.3	6.0 ± 3.6
6b	1.1 ± 0.04	n.p.	0.77	(**) 7.7 ± 1.9	12.2 ± 7.5
6c	1.3 ± 0.03	n.p.	2.1	-	10.2 ± 4.9
6d	1.2 ± 0.05	n.p.	19	(*) 42 ± 0.1	61
5a	13.5 ± 0.4	n.p.	32	(*) 5.5 ± 2.8	>100
10	0.28 ± 0.003	5.3	8.5	(**) 1.4 ± 0.7	92
11	14.7 ± 0.3	n.p.	>50	-	>100

Figure 1.

Viral ribonuclease H-related studies. (A) Compounds tested in antiviral assays. (B) Summary of data from RNase H-related biological studies for HIV and HBV. ^a HIV-1 RT RNaseH IC₅₀ values are reported as the average from triplicate assays ± standard deviation. ^b Concentration of 50% viral replication suppression (EC₅₀) are reported from a single run. n.p. Reflects not protective. ^c CC₅₀ represents concentration at 50% cytotoxicity against the host CEM-SS cells, and are calculated from a single experiment. ^d HBV replication inhibition at 20 μM, unless otherwise defined. ** Indicates that the amount of (+)-strand DNA is <25% and (–)-strand DNA is >60% relative to DMSO control. * Indicates that the amount of (+)-strand DNA is <50% of the amount of (–)-strand DNA relative to DMSO control. – refers to no evidence of selective inhibition of (+)-strand DNA. EC₅₀ values are shown for active compounds, and are reported as the average of two or three dose response curves ± standard deviation. ^e HepDES19 cytotoxicity values are likewise reported as an average of 2–4 assays ± standard deviation.

Figure 2.

HSV-related studies. (A) HSV-1 suppression by synthetic tropolones at concentrations of 5 and 1 μ M. Also shown are clinical anti-HSV agents acyclovir (ACV) and cidofovir (CDV) at 5 μ M. (B) Replication inhibition studies of 3,7-dHTs at 5 μ M. L/R **6a–d** against HSV-2, compound **6a** against 3 HSV-2 clinical isolates, and comparisons of **6a** and ACV against wild-type (WT) and thymidine kinase negative (TK⁻) HSV strains resistant to ACV. (C) EC₅₀ values obtained for **6a** and **6c** against HSV-1 and HSV-2, with data representing the average from 2 dose response curves $\pm$ standard deviation. Also shown are values for ACV

obtained previously by Tavis *et al* under identical conditions.³² CC₅₀ represents concentration at 50% cytotoxicity against the host Vero cells. n.d. = not determined.

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Figure 3. HSV-Associated Assays of Compound 12 and Related Structures. (A) Structures of most potent anti-HSV molecules. (B) Head-to-head comparison of troponoids as HSV-1 viral replication inhibitors at three concentrations. (C) Potency values for **6e** and **12**. Values of **12** were reported previously.¹⁵

Figure 4.

Hypothesized Structural Basis for Potency Increases of **6e** against pUL15. (A) Single orientation of α HT as opposed to (B) two orientations of 3,7-dHTs. (C,D) The structure of the complex between UL15 (PDB id 4IOX) and 3,7-dHT **6e** (green) predicted by molecular docking, showing (C) the biphenyl group of the ligand nested into a hydrophobic groove formed by Lys 640, Asn 583 and Leu 636 and (D) possible π -cation contacts with Lys 640 and close proximity to Asn 583, which could participate in π -NH interactions.

B. Galactose Route (Ōmura, Sunazuka, **2017**)

C. Kojic Acid Route

Scheme 1.

(A) Contiguously hydroxylated tropolones and common mode of metalloenzyme binding. (B) A divergent, unifying galactose approach towards both tropolone classes. (C) Overview of kojic acid route developed by our lab and extended in this study to 3,7-dHTs.

Scheme 2.

(a) Formation of 3,7-dHTs via oxidopyrylium salt **1a** and iodoalkynes **2a** and **2b**. (b) Results from initial attempts at methanol-iodide exchange. (c) Brønsted acid-mediated cycloreversion of oxabicyclic intermediates **3**. (d) Acid-mediated hydrolysis and decarboxylation of **5a**.

Reagents and conditions: (a) *i*-Pr₂NPh, **2a/b**, CH₂Cl₂, 120 °C, 20 min (**3a** = 57%, **3b** = 95%). (b) MeOH/DMAP, 120 °C, 15 min (**4a** = 94%, **4b** = 73%). (c) BCl₃, CH₂Cl₂, r.t., 10 min, (**5a** = 97%, **5b** = 81%). (d) HBr/AcOH, 120 °C, 45 min (**6a** = 55%, **6b** = 87%). (e) DMAP/H₂O, CDCl₃, 100 °C, 90 min, 16%. (f) K₂CO₃/MeOH, r.t., 67%. (g) HBr/AcOH/H₂O, 120 °C, 60 min, 45%.

Scheme 3.

Biogenesis of 6-hydroxytropolones stipitatic acid and stipitonic acid from stipitalide and related 3,7-dHTs.

Scheme 4. Synthesis of 3,7-dHT **6d**.

Reagents and conditions: (a) *i*Pr₂NPh, **2b**, CH₂Cl₂, 100 °C, 1 hr, 5:1 ratio of **3c**:**3b** observed by crude ¹H NMR. (b) NEt₃, CH₂Cl₂, 85%. (c) **2b**, CH₂Cl₂, 100 °C, 1 hr, 71%. (d) DMAP, MeOH, 120 °C, 5 min, 61%. (e) BCl₃, CH₂Cl₂, 0 °C, 5 min, 89%. (f) NaOAc, AcOH, r.t., 15 hr, 93%. (g) NaOH (aq.), r.t., 3 hr, 91%. (h) C₁₈ silica, MeCN/H₂O/TFA, 81%. (i) HBr/AcOH, 120 °C, 45 min, 35%.

Scheme 5.

Synthesis of 3,7-dHT **6e**.

Reagents and conditions: (a) AgNO₃, N-iodosuccinimide, acetone, 0°C, 4 hr, 57%. (b) **2d** and **7a**, CH₂Cl₂, 120°C, 30 min. (c) MeOH/DMAP, 70°C, 15 min, 68% over two steps. (d) BCl₃, CH₂Cl₂, 0 °C, 6 min, 64%. (e) HBr/AcOH, 120°C, 70 min, 70%.