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Synthesis and Evaluation of Bifunctional Anti-HIV Agents Based on Specific CXCR4 Antagonists-AZT Conjugation

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Abstract—We have previously found that T140, a 14-amino acid residue peptide, inhibits infection of target cells by T cell-line-tropic strains of HIV-1 (X4-HIV-1) through its specific binding to a chemokine receptor, CXCR4. Here, we report synthesis and evaluation of bifunctional anti-HIV compounds, which are composed of T140 analogues and a reverse transcriptase inhibitor, 3′-azido-3′-deoxythymidine (AZT). Novel conjugated analogues have been proved to have the ability for controlled release of AZT in neutral aqueous media as well as mouse and feline sera, and high selectivity indexes (SIs, 50% cytotoxic concentration/50% effective concentration) caused by a synergistic effect of two different regenerating agents. Thus, these bifunctional compounds have several potential advantages. T140 analogues can possibly work as a carrier of AZT targeting T cells due to their specific affinity for CXCR4 on T cells. A synergistic effect by two types of regenerating agents may enable drug dosage to be reduced, and thus it may effectively suppress toxic side effects and the appearance of drug-resistant virus.

Introduction

Recently, 'highly active anti-retroviral therapy (HAART)', which involves a combination of reverse transcriptase/protease inhibitors, has dramatically improved the clinical treatment of individuals with AIDS or HIV infection.[1] However, this combination therapy using multi-types of anti-HIV drugs has not yet reached the stage of perfection owing to several serious problems including the emergence of viral strains with multi-drug resistance, significant side effects and high costs. An ideal therapeutic approach would involve efficient delivery of anti-HIV agents to target cells. The discovery of chemokine receptors, CXCR4 and CCR5, as coreceptors for T cell line-tropic HIV-1 (X4-HIV-1)[2] and macrophage-tropic HIV-1 (R5-HIV-1),[3] respectively, provides valuable access to such a strategy. It is desirable that the conjugation of specific chemokine receptor antagonists with another anti-HIV agent renders efficient drug delivery to target cells. Several chemokine receptor antagonists have been reported by us and others.[4] We demonstrated that a synthetic peptide analogue, T22 ([Tyr,[5,12] Lys[7]]-polyphemusin II), is a CXCR4 antagonist that blocks X4-HIV-1 entry mediated by this coreceptor [anti-HIV activity: 50% effective concentration (EC_{50}) = 80 nM, antagonism of entry by X4-HIV-1: EC_{50} = 5.1 nM].[4a,b] T22 is an 18-residue peptide amide with two intrachain disulfide bonds, which was derived from chemical modifications of horseshoe crab self-defense peptides, tachyplesin and polyphemusin (Fig. 1). Thus, we previously synthesized bifunctional anti-HIV compounds, which are composed of a T22 analogue, T131 (des-[Cys[8,13], Tyr[9,12]]-[Tyr[3], D-Lys,[10] Pro[11]]-T22 lacking the C-terminal amide) (Fig. 1), and 3′-azido-3′-deoxythymidine (AZT).[5] T131 was employed as a carrier of AZT to target T cells owing to its binding affinity for CXCR4. It was our expectation that AZT would be released from the AZT-T131 conjugate on the T cell surface followed by the T-cell uptake via nucleoside transport systems.[6] AZT is a reverse transcriptase inhibitor, which has been used frequently in clinical therapy.[7] As a result, AZT and the T131 derivative exhibited a synergistic effect for anti-HIV activity in vitro. Kiso et al. reported the synthesis and biological evaluation of anti-HIV double-drugs, which are composed of two representative classes of

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anti-HIV agents: HIV protease inhibitors and reverse transcriptase inhibitors.[8] Our bifuctional anti-HIV compounds are thought to target T cells based on CXCR4 antagonists, which have binding affinity for the HIV coreceptor. However, T131 is not a fairly strong anti-HIV agent (anti-HIV activity: $EC_{50} = 74$ nM, antagonism of entry by X4-HIV-1: $EC_{50} = 31$ nM). Recently, we found a more potent CXCR4 antagonist, T140 ([L-3-(2-naphthyl)-alanine (Nal),[3] L-citrulline (Cit)[12]]-T131), which has the highest level of HIV-1 inhibition activity ($EC_{50} = 3.5$ nM) based on antagonism of entry by X4-HIV-1 ($EC_{50} = 0.43$ nM) (Fig. 1).[9] In this report, we synthesized bifunctional anti-HIV agents, which are composed of T140 and AZT, investigated their behaviour in aqueous media or serum in detail, and evaluated their anti-HIV activity in vitro. We also discuss a comparative study with a combined assay using an equimolar mixture of two agents, such as AZT and T140 (or a modified T140 derivative, which is to be released from the parent conjugated compound). According to our very recent study, T140 is not stable in mouse and feline sera due to cleavage of the C-terminal Arg^{14}, whereas the C-terminally amidated analogue of T140 (**TZ14004**, Fig. 1) is completely stable (unpublished data). Since Arg^{14} is an indispensable residue for anti-HIV activity,[10] the C-terminally amidated version of the above T140-related compounds was similarly synthesized and evaluated.

Results and Discussion

Chemistry

The structures of synthetic T140 analogues including compounds conjugated with AZT are shown in Fig. 1 and Schemes 1–3. T140 was previously synthesized using *p*-benzyloxybenzyl alcohol (Alko)-resin.[9] In the synthesis of an N^{α}-hemisuccinate derivative of T140 (**TZ14001**), the protected T140-resin, which was constructed by 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase synthesis, was treated with succinic anhydride in pyridine (after cleavage of an N^{α}-Fmoc-group) to yield the protected TZ14001-resin (Scheme 1). Then, cleavage from the resin and deprotection of all protecting groups were performed simultaneously with disulfide bond formation by treatment with the TMSCl-DMSO/TFA system in a one-pot manner[5] in order to obtain **TZ14001**. In the synthesis of an N^{α}-succinimide derivative of T140 (**TZ14002**), *mono*-methyl succinate was condensed on the N^{α}-amino group of the protected T140-resin using 1,3-diisopropylcarbodiimide (DIPCDI) and *N*-hydroxybenzotriazole (HOBt), followed by deprotection/cleavage with TMSBr treatment to yield an N^{α}-*mono*-methyl succinate derivative of [$Cys(SH)^{4,13}$]-T140 (Scheme 2). Then, the crude Cys(SH)-peptide was air-oxidized in the NH_4OAc aqueous solution at pH 7.8, with simultaneous succinimide formation, to yield **TZ14002**. In the synthesis of a conjugate compound AZT-Suc-T140 (**TZ14003**), AZT-5′-hemisuccinate, which was previously prepared,[5] was condensed on the N^{α}-amino group of the protected T140-resin using DIPCDI and HOBt to yield the protected TZ14003-resin (Scheme 3). Then, cleavage/deprotection was performed simultaneously with disulfide bond formation by treatment with the TMSCl-DMSO/TFA system in a one-pot manner in order to obtain **TZ14003**. The conjugated compound **TZ14003** is not stable in aqueous media at pH 7–8, such as the air-oxidation condition, due to a release of AZT from the peptide by the concomitant formation of the N^{α}-succinimide derivative of T140 (see the following section). Thus, the usual air-oxidizing reaction condition cannot be used for the synthesis of **TZ14003**. For deprotection/cleavage and disulfide-bond formation in the synthesis of **TZ14003**, we employed the above one-pot procedure using the TMSCl-DMSO/TFA system, which is performed in acidic media without the undesired succinimide formation. C-Terminally amidated analogues of T140, **TZ14001**, **TZ14002** and **TZ14003** (**TZ14004**, **TZ14005**, **TZ14006** and **TZ14007**, respectively) were prepared using 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)-valeric acid resin (PAL^{TM}-resin) instead of Alko-resin in the same way as in the synthesis of T140, **TZ14001**, **TZ14002** and **TZ14003**, respectively.

Investigation on the behaviour of conjugated compounds in aqueous media or serum

At first, we investigated the behaviour of a C-terminally carboxy-free conjugate AZT-Suc-T140 (**TZ14003**) in a phosphate buffer (pH 7.4) at 37 °C. As depicted in Scheme 4, a release of AZT by the concomitant formation of an N^{α}-succinimide derivative of T140 (**TZ14002**) was confirmed by HPLC analysis and ion-spray mass spectrometry (IS-MS) (Fig. 2a). Subsequently, hydrolysis of **TZ14002** yielded an N^{α}-hemisuccinate derivative of T140 (**TZ14001**). Since a parent conjugated compound is thought to be disintegrated according to first-order kinetics, a half-life is estimated by linearization of plots of a logarithm of concentration versus time: $t_{(1/2)} = 2.5$ h. The disintegration of a C-terminally amidated conjugate AZT-Suc-TZ14004 (**TZ14007**) occurred faster than that of **TZ14003**, through the same pathway (Fig. 2b). Succinimide **TZ14006** (35.0%) and succinate **TZ14005** (23.4%) appeared at 0 h straight after dissolution of **TZ14007** into a phosphate buffer. Linearization

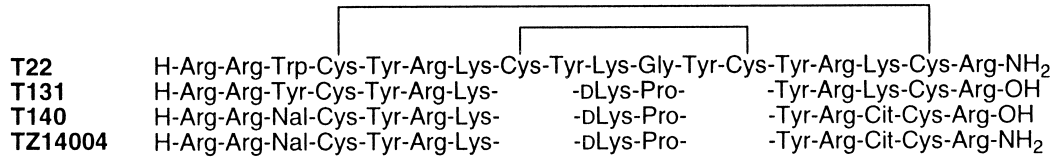
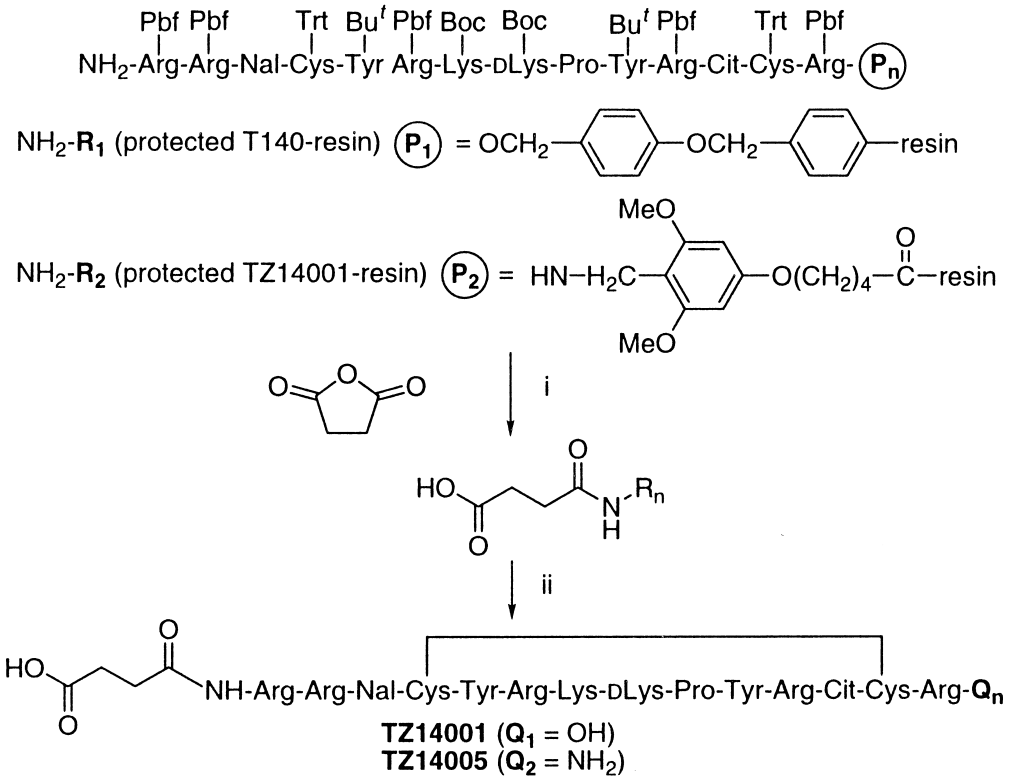
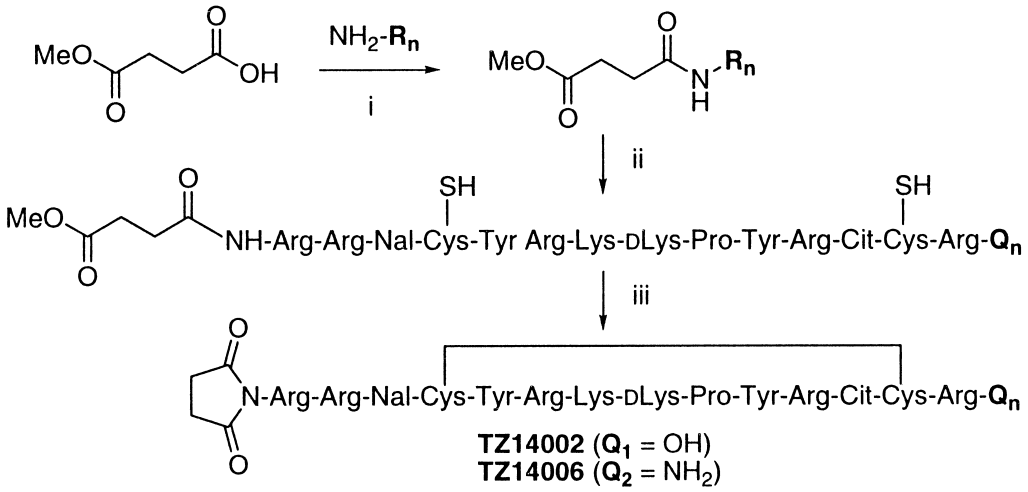


Figure 1. Amino acid sequences of T22, T131, T140 and **TZ14004**, with alignment based on their homology. The disulfide linkages are shown by solid lines. T22 have two disulfide linkages between Cys^4 and Cys^{17} and between Cys^8 and Cys^{13} whereas T131, T140 and **TZ14004** have one disulfide linkage between Cys^4 and Cys^{13}.



Scheme 1. Reagents: (i) pyridine; (ii) TMSCl-DMSO/TFA (α-NH of Arg[1] is intentionally shown for easy understanding).



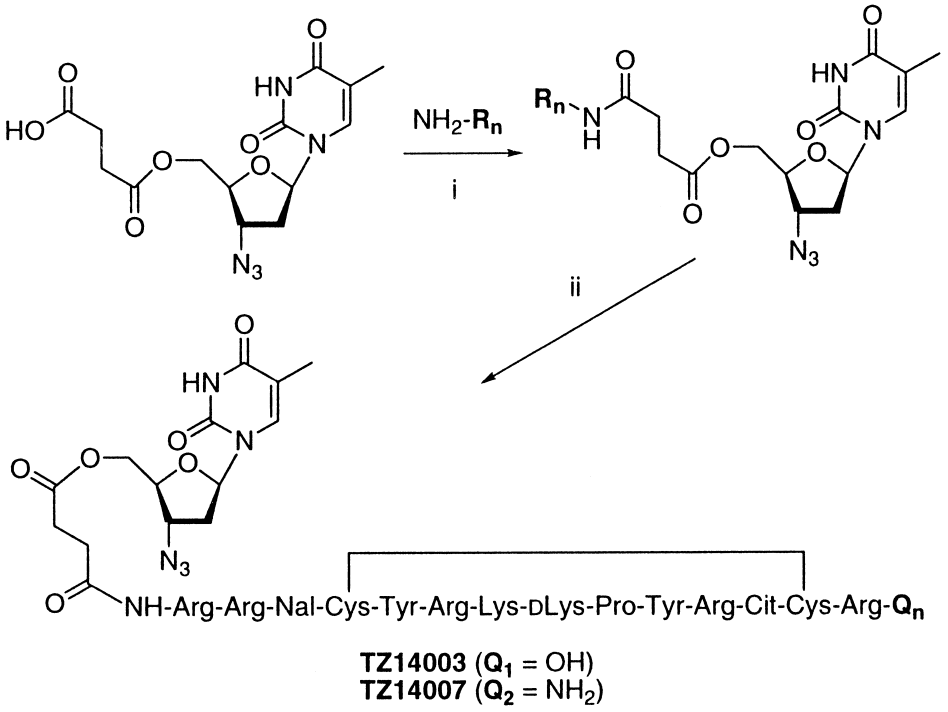
Scheme 2. Reagents (i) DIPCDI, HOBt; (ii) 1 M TMSBr-thioanisole/TFA; (iii) 0.05 M aq $AcONH_4$, pH 7.8.

of plots of a logarithm of concentration versus time could be performed without any problem to yield a half-life: $t_{(1/2)} = 1.9$ h. We cannot explain why succcinimide and succinate derivatives appeared as soon as **TZ14007** was dissolved in the phosphate buffer. These results demonstrated that both conjugated compounds can efficiently release AZT in aqueous assay media at pH 7.4. Next, the behaviour of AZT-Suc-TZ14004 (**TZ14007**) in mouse and feline sera was examined at 37 °C. The disintegration of **TZ14007** in mouse and feline sera occurred much faster than that in the phosphate buffer (Fig. 2c and d): mouse, $t_{(1/2)} = 16.2$ min; feline, $t_{(1/2)} = 33.3$ min. Incubation of AZT-Suc-T140 (**TZ14003**) with mouse and feline sera yielded not only AZT, **TZ14002**, **TZ14001**, but also C-terminally truncated derivatives of **TZ14003**, **TZ14002** and **TZ14001**, which lack Arg[14] (data not shown). These results suggested that the conjugated compounds can release AZT in physiological conditions although C-terminal Arg[14] of T140 is not stable in AZT-Suc-T140 (**TZ14003**).

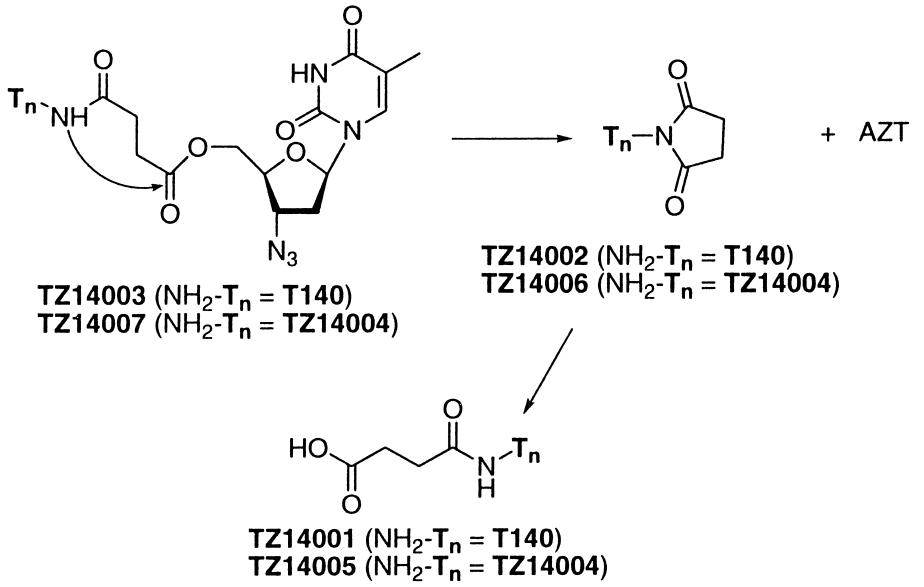
Anti-HIV activity and cytotoxicity

The anti-HIV activity and cytotoxicity of T140 analogues, their AZT-conjugates and equimolar mixtures of two agents are summarized in Table 1. In aqueous assay

media (pH 7.4), conjugated compounds (**TZ14003** and **TZ14007**) release AZT and N^{α}-succinimide derivatives of T140 and **TZ14004** (**TZ14002** and **TZ14006**), the latter of which are successively converted into the corresponding N^{α}-succinate derivatives (**TZ14001** and **TZ14005**). Thus, we examined the anti-HIV activity and cytotoxicity of a single compound of N^{α}-succinate and N^{α}-succinimide derivatives (**TZ14001**, **TZ14005**, **TZ14002** and **TZ14006**) (Table 1, entries 2, 3, 10, and 11). It is thought that N^{α}-succinimide derivatives are partially converted into N^{α}-succinate derivatives in aqueous assay media. However, this phenomenon is intrinsically inevitable. Furthermore, a comparative assay of an equimolar mixture of AZT and an N^{α}-succinate or N^{α}-Succinimide derivative was performed (entries 6–8 and 13–15). The examination of C-terminally carboxy-free compounds is shown in entries 1–4 and 6–8. N^{α}-Succinate and N^{α}-succinimide derivatives of T140 (**TZ14001** and **TZ14002**) showed 100-fold lower anti-HIV activity than T140 (entries 1–3), suggesting that an addition of a succinate or succinimide group at the N-terminus is not suitable for high anti-HIV activity. However, an equimolar mixture of AZT and **TZ14001** or **TZ14002** (molar ratio 1:1) caused a remarkable increase in anti-HIV activity (entries 7 and 8), compared to a single compound, such as **TZ14001**,



Scheme 3. Reagents: (i) DIPCDI, HOBt; (ii) TMSCl-DMSO/TFA.



Scheme 4. Disintegration pathway of conjugated compounds in phosphate buffer at pH 7.4.

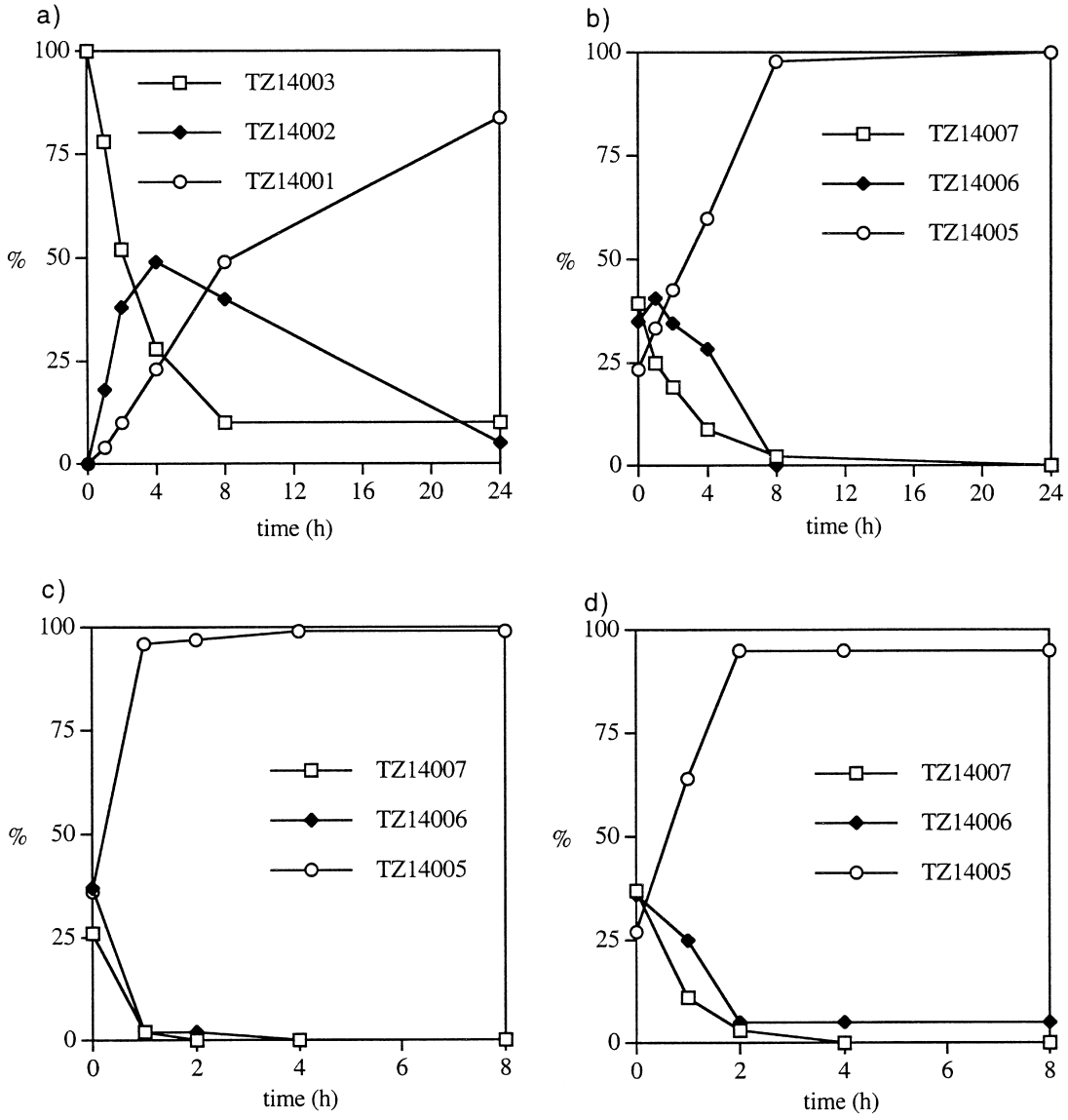


Figure 2. Behaviour of conjugated compounds in phosphate buffer at pH 7.4 and mouse/feline serum. HPLC peak areas (%) of starting materials and generated products were shown with the passage of time (h): (a) **TZ14003** in phosphate buffer; (b) **TZ14007** in phosphate buffer; (c) **TZ14007** in mouse serum; (d) **TZ14007** in feline serum.

TZ14002. This increased activity might be explained by a synergistic effect of AZT and **TZ14001**/**TZ14002**. A conjugated compound AZT-Suc-T140 (**TZ14003**) also showed a remarkable increase in anti-HIV activity (entry 4), compared to a single compound. **TZ14001** and **TZ14002** might coexist mainly with AZT in assay media of **TZ14003** during 5 days' incubation. It is reasonable that an EC_{50} value of **TZ14003** is between the values of **TZ14001**/AZT (1:1) and **TZ14002**/AZT (1:1). An equimolar mixture of AZT and T140 showed the highest anti-HIV activity (entry 6). Taken together, the daughter species, **TZ14001** and **TZ14002**, have moderate potency, whereas AZT-Suc-T140 (**TZ14003**) shows relatively high anti-HIV activity. None of the C-terminally carboxy-free compounds have significant cytotoxicity (50% cytotoxic concentration (CC_{50}) $>20\,\mu M$, entries 1–4 and 6–8). The examination of C-terminally amidated compounds is shown in entries 9–15. A C-terminally amidated analogue of T140 (**TZ14004**) showed lower anti-HIV activity than T140 (entry 9). N^{α}-Succinate and N^{α}-succinimide derivatives (**TZ14005** and **TZ14006**) also showed slightly lower activity than **TZ14001** and **TZ14002** (entries 10 and 11). An equimolar mixture of AZT and **TZ14005** or **TZ14006** (1:1) and a conjugated compound AZT-Suc-TZ14004 (**TZ14007**) also showed a remarkable increase in anti-HIV activity (entries 12, 14, and 15), compared to each single compound, such as **TZ14005**, **TZ14006**. **TZ14005** and **TZ14006** might coexist mainly with AZT in assay media of **TZ14007** in the same manner as in media of **TZ14003**. An EC_{50} value of **TZ14007** is reasonably between the values of **TZ14005**/AZT (1:1) and **TZ14006**/AZT (1:1). An equimolar mixture of AZT and **TZ14004** showed modest anti-HIV activity (entry 13), possibly due to low potency of **TZ14004**. According to our previous SAR study, C-terminally amidated analogues of T140 derivatives have stronger cytotoxicity than the corresponding C-terminally carboxy-free compounds.[11] In this

Table 1. Anti-HIV activity and cytotoxicity of T140 analogues, their AZT-conjugates and equimolar mixtures of two agents

Entry	Compound	EC_{50} (nM)	CC_{50} (μM)	SI
1	T140	3.3	>20	>6100
2	**TZ14001**	310	>20	>65
3	**TZ14002**	330	>20	>61
4	**TZ14003**	4.6	>20	>4300
5	AZT	20	150	7500
6	AZT: T140	1.2	>20	>17,000
7	AZT : **TZ14001**	13	>20	>1600
8	AZT : **TZ14002**	3.2	>20	>6300
9	**TZ14004**	68	8	120
10	**TZ14005**	730	>20	>27
11	**TZ14006**	390	>20	>51
12	**TZ14007**	6.1	13	2200
13	AZT : **TZ14004**	11	7	610
14	AZT : **TZ14005**	7.9	11	1400
15	AZT : **TZ14006**	4.0	9	2300

EC_{50} values are the concentrations for 50% protection of HIV-induced cytopathogenicity in MT-4 cells. CC_{50} values are based on the reduction of the viability of mock-infected cells. SI is shown as CC_{50}/EC_{50}. All data are mean values for at least three experiments.

study, all the C-terminally amidated compounds except for **TZ14005** and **TZ14006** have also stronger cytotoxicity than the corresponding C-terminally carboxy-free compounds (entries 9–15). **TZ14005** and **TZ14006** did not show any significant cytotoxicity upto $CC_{50} = 20\ \mu M$. These results suggest that AZT-Suc-TZ14004 (**TZ14007**) is an effective conjugated compound, and that, in terms of stability in physiological conditions such as in serum, **TZ14007** is superior to AZT-Suc-T140 (**TZ14003**).

These conjugated compounds have several potential advantages. (1) These compounds have binding affinity for an HIV coreceptor CXCR4, and T140 derivatives can possibly work as a drug carrier targeting T cells. (2) There are practical possibilities of development of effective prodrugs of AZT based on AZT-T140 analogue conjugates that possess the potency of a controlled release of AZT. (3) These conjugates are bifunctional anti-HIV agents, which have two different actions. Thus, the expectable synergistic effect would enable drug dosage to be reduced, and effectively suppress the emergence of drug-resistant viral strains and significant side effects. Although these hypotheses have not yet been proven completely up to now, the conjugates, which were synthesized in this study, exhibited high anti-HIV activity and selectivity indexes, and the potency of an efficient release of AZT in vitro. It is a matter of course that the application of this strategy to conjugates with anti-HIV agents other than AZT would likewise be possible. In future, we must investigate whether the conjugates can practically target and reach CXCR4 on T cells in vivo. Accordingly, modification and refinement of the conjugates including a succinyl linker might be required. Cleavage rates of linkers should be suitably adjusted to physiological and clinical conditions. Taken together, our present results will be useful as basic information for developing new types of bifunctional anti-HIV drugs based on CXCR4 antagonists–anti-HIV agents conjugation.

Experimental

HPLC solvents were H_2O and CH_3CN, both containing 0.1% (v/v) TFA. For analytical HPLC, a Cosmosil 5C18-AR column (4.6×250 mm, Nacalai Tesque Inc., Kyoto, Japan) was eluted with a linear gradient of CH_3CN at a flow rate of 1 mL/min on a Waters LC Module I equipped with a Waters 741 Data Module (Nihon Millipore, Ltd, Tokyo, Japan). Preparative HPLC was performed on a Waters Delta Prep 4000 equipped with a Cosmosil 5C18-AR column (20×250 mm, Nacalai Tesque Inc.) using a linear gradient of CH_3CN at a flow rate of 7 mL/min. For gel filtration, the solution was applied to a column of Sephadex G-15 (2.1×30 cm), which was eluted with 1 M AcOH. Amino acid analysis was conducted using a Hitachi 835 or L-8500 instrument (Tokyo, Japan). Ion-spray (IS)-mass spectrum was obtained with a Sciex API*III*E triple quadrupole mass spectrometer (Toronto, Canada). Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)-mass spectrum was obtained with a Shimadzu KRATOS analytical mass spectrometer (Kyoto, Japan). Optical rotation of a peptide in aqueous solution was measured with a JASCO DIP-360 digital polarimeter (Tokyo, Japan) or a Horiba high-sensitive polarimeter SEPA-200 (Kyoto, Japan). Fmoc-protected amino acids and Alko-resin were purchased from Watanabe Chemical Industries, Ltd (Hiroshima, Japan) or Calbiochem-Novabiochem Japan, Ltd (Tokyo, Japan). PAL™-resin was purchased from Millipore. All the other chemicals were purchased from either Nacalai Tesque Inc. or Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Synthesis of N^{α}-hemisuccinate-T140 (TZ14001)

The protected T140-resin was manually constructed using Fmoc-based solid-phase synthesis on Fmoc-Arg(Pbf)-Alko-resin (0.35 meq/g, 0.048 mmol scale, Pbf = 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl). Fmoc-protected amino acid derivatives (2.5 equiv) were successively condensed using DIPCDI (2.5 equiv) in the presence of HOBt (2.5 equiv). The following side-chain protecting groups were used: Pbf for Arg, Trt for Cys, Bu^t for Tyr and Boc for Lys and D-Lys. The resulting protected T140 resin (160 mg, 24 μmol) was treated with succinic anhydride (6.09 mg, 60.0 μmol) in pyridine (3 mL) at room temperature for 2 h. The resulting protected N^{α}-hemisuccinate-T140 resin was treated with TMSCl (210 μL, 1.66 mmol) in TFA (23 mL) in the presence of anisole (300 μL) at room temperature. After 1 h, DMSO (3.80 mL, 53.5 mmol) was added to the reaction mixture at 4 °C and reaction was allowed to continue for 1 h. After removal of the resin by filtration, ice-cold dry diethyl ether (30 mL) was added to the filtrate. The resulting powder was collected by centrifugation and then washed three times with ice-cold dry diethyl ether (20 mL×3). The crude product was purified by preparative HPLC and gel-filtration to afford a fluffy white powder of N^{α}-hemisuccinate-T140 (**TZ14001**); yield 10.5 mg [4.01 μmol, 16.7% calculated from the Fmoc-Arg(Pmc)-Alko-resin].

Amino acid ratios after hydrolysis with 6 M HCl (values in parentheses are theoretical): Cit not determined (1), cystine not determined (1), Tyr 2.00 (2), Lys and D-Lys 2.00 (2), Nal not determined (1), Arg 4.56 (5) and Pro 1.04 (1). IS-MS (reconstructed) Found: *m/s* 2137.0 (calcd for $C_{94}H_{145}N_{33}O_{21}S_2$: 2137.5). $[\alpha]_D^{29} = -34.65°$ (*c* 0.1, H_2O).

Synthesis of N$^{\alpha}$-succinimide-T140 (TZ14002)

The protected T140-resin (160 mg, 24 μmol), which was constructed in the synthesis of **TZ14001**, was treated with *mono*-methyl succinate (7.93 mg, 60.0 μmol), DIPCDI (9.39 μL, 60.0 μmol) and HOBt (9.19 mg, 60.0 μmol) in DMF (3 mL) at room temperature for 2 h. The resulting protected N$^{\alpha}$-*mono*-methyl succinate-T140 resin was treated with 1 M TMSBr-thioanisole/TFA (10 mL) in the presence of *m*-cresol (488 μL, 200 equiv)-1,2-ethanedithiol (EDT) (200 μL, 100 equiv) at 0 °C for 1 h. After removal of the resin by filtration, the filtrate was concentrated and ice-cold dry diethyl ether (30 mL) was added to the filtrate. The resulting powder was collected by centrifugation, washed three times with ice-cold dry diethyl ether (20 mL×3), and then dissolved in 50% AcOH (2 mL). Subsequently, the solution was diluted to total volume 300 mL with H_2O, and pH was then adjusted to 7.8 with concentrated NH_4OH at 0 °C. After air-oxidation at room temperature for 1 day, the crude product in the solution was purified by preparative HPLC and gel filtration to afford a fluffy white powder of N$^{\alpha}$-succinimide-T140 (**TZ14002**); yield 2.55 mg [1.03 μmol, 4.28% calculated from the Fmoc-Arg(Pmc)-Alko-resin].

Amino acid ratios after hydrolysis with 6 M HCl (values in parentheses are theoretical): Cit not determined (1), cystine not determined (1), Tyr 2.00 (2), Lys and D-Lys 2.16 (2), Nal not determined (1), Arg 4.83 (5) and Pro 0.95 (1). TOF-MS Found: $(M+H)^+$, 2120.9 ($C_{94}H_{143}N_{33}O_{20}S_2$ requires M + H, 2120.5). $[\alpha]_D^{28} = -177.77°$ (*c* 0.1, H_2O).

Synthesis of AZT-Suc-T140 (TZ14003)

The protected T140-resin (200 mg, 50 μmol), which was constructed using Fmoc-Arg(Pbf)-Alko resin (0.64 meq/g, 0.05 mmol scale) in the same way as in the synthesis of **TZ14001**, was treated with AZT-5′-hemisuccinate (3′-azido-3′-deoxythymidine 5′-hemisuccinate)[5] (92.9 mg, 250 μmol), DIPCDI (39.1 μL, 250 μmol) and HOBt (38.3 mg, 250 μmol) in DMF (3 mL) at room temperature for 2 h. The resulting protected AZT-Suc-T140 resin was treated with TMSCl (420 μL, 3.32 mmol)/TFA (46 mL)-anisole (600 μL) and DMSO (7.60 mL, 107 mmol) in the same way as in the synthesis of **TZ14001** to afford a fluffy white powder of AZT-Suc-T140 (**TZ14003**); yield 20.0 mg (7.13 μmol, 14.3% calculated from the Fmoc-Arg(Pmc)-Alko resin).

Amino acid ratios after hydrolysis with 6 M HCl (values in parentheses are theoretical): Cit not determined (1), cystine not determined (1), Tyr 1.93 (2), Lys and D-Lys 2.14 (2), Nal not determined (1), Arg 5.02 (5) and Pro 1.00 (1). IS-MS (reconstructed) Found: *m/s* 2386.7 (calcd for $C_{104}H_{156}N_{38}O_{24}S_2$: 2386.8). $[\alpha]_D^{28} = -30.58°$ (*c* 0.9, H_2O).

Synthesis of the C-terminally amidated analogue of T140 (TZ14004)

The protected TZ14004-resin was constructed on the PAL^{TM} resin (0.36 meq/g, 0.1 mmol scale) in the same way as in the construction of the protected T140 resin. The resulting protected TZ14004 resin (100 mg, 14 μmol) was similarly treated with 1 M TMSBr-thioanisole/TFA (5 mL)-*m*-cresol (244 μL, 170 equiv)-EDT (100 μL, 85 equiv) and then air-oxidized in the same way as in the synthesis of **TZ14002** to afford a fluffy white powder of **TZ14004**; yield 4.84 mg (1.88 μmol, 13.3% calcd from the PAL^{TM} resin).

Amino acid ratios after hydrolysis with 6 M HCl (values in parentheses are theoretical): Cit not determined (1), cystine not determined (1), Tyr 2.00 (2), Lys and D-Lys 2.30 (2), Nal not determined (1), Arg 5.16 (5) and Pro 1.21 (1). IS-MS (reconstructed) Found: *m/s* 2035.7 (calcd for $C_{90}H_{142}N_{34}O_{17}S_2$: 2036.4). $[\alpha]_D^{24} = -130.43°$ (*c* 0.2, H_2O).

Synthesis of N$^{\alpha}$-hemisuccinate-TZ14004 (TZ14005)

The protected TZ14004-resin (170 mg, 24 μmol) was treated with succinic anhydride (6.09 mg, 60.0 μmol)/pyridine (3 mL), and the resulting protected N$^{\alpha}$-hemisuccinate-TZ14004 resin was then treated with TMSCl (210 μL, 1.66 mmol)/TFA (23 mL)-anisole (300 μL) and DMSO (3.80 mL, 53.5 mmol) in the same way as in the synthesis of **TZ14001** to afford a fluffy white powder of N$^{\alpha}$-hemisuccinate-TZ14004 (**TZ14005**); yield 6.04 mg (2.36 μmol, 9.85% calculated from the PAL^{TM} resin).

Amino acid ratios after hydrolysis with 6 M HCl (values in parentheses are theoretical): Cit not determined (1), cystine not determined (1), Tyr 2.00 (2), Lys and D-Lys 2.25 (2), Nal not determined (1), Arg 4.94 (5) and Pro 1.16 (1). IS-MS (reconstructed) Found: *m/s* 2137.4 (calcd for $C_{94}H_{146}N_{34}O_{20}S_2$: 2136.5). $[\alpha]_D^{25} = -119.40°$ (*c* 0.2, H_2O).

Synthesis of N$^{\alpha}$-succinimide-TZ14004 (TZ14006)

The protected TZ14004-resin (170 mg, 24 μmol) was treated with *mono*-methyl succinate (7.93 mg, 60.0 μmol), DIPCDI (9.39 μL, 60.0 μmol) and HOBt (9.19 mg, 60.0 μmol) in DMF (3 mL) in the same way as in the synthesis of **TZ14002**. The resulting protected N$^{\alpha}$-*mono*-methyl succinate-TZ14004-resin was treated with 1 M TMSBr-thioanisole/TFA (10 mL)-*m*-cresol (488 μL, 200 equiv)-EDT (200 μL, 100 equiv), and then air-oxidized in the same way as in the synthesis of **TZ14002** to afford a fluffy white powder of N$^{\alpha}$-succinimide-TZ14004 (**TZ14006**); yield 6.94 mg (2.67 μmol, 11.1% calculated from the PAL^{TM} resin).

Amino acid ratios after hydrolysis with 6 M HCl (values in parentheses are theoretical): Cit not determined (1), cystine not determined (1), Tyr 2.00 (2), Lys and D-Lys 2.28 (2), Nal not determined (1), Arg 5.08 (5) and Pro 1.09 (1). TOF-MS Found: $(M+H)^+$, 2121.5 ($C_{94}H_{144}N_{34}O_{19}S_2$ requires M + H, 2119.5). $[\alpha]_D^{27} = -115.87°$ (*c* 0.2, H_2O).

Synthesis of AZT-Suc-TZ14004 (TZ14007). The protected TZ14004-resin (170 mg, 24 μmol) was treated with AZT-5′-hemisuccinate (43.9 mg, 120 μmol), DIPCDI (18.8 μL, 120 μmol) and HOBt (18.3 mg, 120 μmol) in DMF (1.5 mL) in the same way as in the synthesis of **TZ14003**. The thus-formed protected AZT-Suc-TZ14004-resin was treated with TMSCl (210 μL, 1.66 mmol)/TFA (23 mL)-anisole (300 μL) and DMSO (3.80 mL, 53.5 mmol) to afford a fluffy white powder of AZT-Suc-TZ14004 (**TZ14007**); yield 5.63 mg (1.96 μmol, 8.18% calculated from the PAL™ resin).

Amino acid ratios after hydrolysis with 6 M HCl (values in parentheses are theoretical): Cit not determined (1), cystine not determined (1), Tyr 2.00 (2), Lys and D-Lys 2.04 (2), Nal not determined (1), Arg 4.73 (5) and Pro 1.08 (1). TOF-MS Found: $(M+H)^+$, 2387.3 ($C_{104}H_{157}N_{39}O_{23}S_2$ requires M + H, 2386.8). $[\alpha]_D^{28} = -122{,}99°$ (*c* 0.2, H_2O).

Behaviour of conjugated compounds in phosphate buffer at pH 7.4

Conjugated compounds (100 nmol) was dissolved in phosphate buffer (10 mM, pH 7.4, 200 μL), and incubated at 37 °C. At intervals (0, 1, 2, 4, 8, 24 h), an aliquot (8 μL) was sampled and examined by analytical HPLC with a linear gradient of CH_3CN (10–50%, 30 min). HPLC peaks of the starting compounds and generated products including AZT were identified by IS-MS, and their amounts were quantitated from the peak areas.

Behaviour of conjugated compounds in mouse or feline serum

Conjugated compounds (100 nmol) was dissolved in mouse or feline serum (100 μL)/H_2O (100 μL), and incubated at 37 °C. At intervals, an aliquot was sampled and examined by analytical HPLC in the same way as in the behaviour in phosphate buffer.

Estimation of half-lives of conjugated compounds in phosphate buffer or serum

Disintegration of conjugated compounds in phosphate buffer or serum is thought to be performed theoretically by first-order kinetics according to the equation

$$C = C_0 \cdot \exp(-kt)$$

where C and C_0 are the concentrations of the parent compounds at time t and 0, respectively, and k is the rate constant. This equation is converted into

$$\ln C = \ln C_0 - kt$$

The slope of the plots of $\ln C$ versus t corresponds to the k values. The half-life [$t_{(1/2)}$] of this reaction can be obtained as

$$t_{(1/2)} = \ln 2/k$$

Cell culture

Human T-cell lines, MT-4 and MOLT-4 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin.

Virus

A strain of X4-HIV-1, HIV-1_{IIIB}, was used for the anti-HIV assay. This virus was obtained from the culture supernatant of HIV-1 persistently infected MOLT-4/HIV-1_{IIIB} cells, and stored at −80 °C until used.

Anti-HIV-1 assay

Anti-HIV-1 activity was determined based on the protection against HIV-1-induced cytopathogenicity in MT-4 cells. Various concentrations of test compounds were added to HIV-1-infected MT-4 cells at a multiplicity of infection (MOI) of 0.01, and placed in wells of a flat-bottomed microtiter tray (1.5×10^4 cells/well). After 5 days' incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (EC_{50}).[12] Cytotoxicity of compounds was determined based on the viability of mock-infected cells using the MTT method (CC_{50}).

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